REMARKS

This document is submitted in response to the Office Action dated December 18, 2006 ("Office Action").

Applicants have amended claim 20, to replace "adverse reactions" with "Steven-Johnson syndrome or toxic epidermal necrolysis in response to carbamazepine." Support for this amendment can be found at, e.g., in original claims 2 and 5. Applicants have also amended claims 1 and 20 to remove non-elected subject matter. Further, Applicants have added new claim 26, support for which appears, for example, in Table 1, page 30.[1] Finally, Applicants have cancelled claims 3, 6, and 21.

Upon entry of the amendments, claims 1, 8-12, 20, and 22-26 will be under examination. Applicants respectfully request that the Examiner reconsider this application in view of the following remarks.

Claim Objections

Claims 1, 12, and 20 are objected to for reciting subject matter that is not elected. See the Office Action, pages 2-3, section 1. Applicants have deleted the non-elected subject matter from claims 1 and 20. Applicants, however, disagree that non-elected species should be removed from claim 12. Please note that claim 12 recites a Markush group that includes six species of equivalent genetic marker of HLA-B*1502, e.g., the elected species HLA-Cw*0801. MPEP § 803.02 requires that the patentability of the elected species be examined first and if it is found allowable, the examination should be extended to the non-elected species. Applicants thus submit that it is not necessary to remove the non-elected species from claim 12 at this point.

Claim Rejection under 35 U.S.C. § 112, First Paragraph (Enablement)

Claims 1, 3, 6, 8-12, and 20-25 stand rejected for lack of enablement on four grounds.

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[1] Table 1 shows the frequency of HLA-B*1502 in 42 Taiwanese patients with carbamazepine induced Stevens-Johnson syndrome/toxic epidermal necrolysis. As Taiwanese are Mongoloids or descendents of Mongoloids, Table 1 supports new claim 26.

Among the rejected claims, claims 3, 6, and 21 have been cancelled. Applicants will traverse each of the grounds below.

I

Apparently referring to claims 20-25, the Examiner asserts that the Specification does not "reasonably provide enablement for assessing the risk of **any** other adverse reactions in response to **any** other drugs." See the Office Action, page 3, third paragraph; emphases added. Note that claim 21 has been cancelled.

Applicants have amended claim 20 to limit it to only one adverse drug reaction, i.e., Stevens-Johnson Syndrome (SJS)/toxic epidermal necrolysis (TEN), and only one drug, i.e., carbamazepone (CBZ). It is submitted that the amendments have rendered moot the Examiner's ground for rejecting claim 20, as well as claims 22-25 dependent from it.

II

The Examiner asserts that the Specification is not enabling for assessing the risk of adverse drug reactions in any human population other than Taiwanese. See the Office Action, page 3, third paragraph. Applicants respectfully disagree.

Independent claim 1 is discussed first. This claim is drawn to a method of assessing the risk of a patient for developing SJS/TEN in response to CBZ by detecting the presence of HLA-B*1502.

Citing Lonjou et al., Alfirevic et al., and Hung et al., the Examiner rejects claim 1 on the ground that: [i]t is unpredictable as to whether or not HLA-B*1502 would be indicative of risk of an adverse drug reaction in another population." See the Office Action, page 6, last paragraph, and page 11, second paragraph, through page 12, first paragraph. Applicants disagree.

Applicants submit that the Examiner has merged two distinct concepts: HLA-B*1502 being a **risk indicator** of a patient (required by claim 1) and HLA-B*1502 being a **prediction marker** in a population (discussed in the cited references). As a risk indicator, HLA-B*1502 is used to determine whether **a specific patient** is at risk for CBZ-induced SJS/TEN. As such, HLA-B*1502 only needs to be associated with this adverse drug reaction. On the other hand, being a prediction marker, HLA-B*1502 is used to identify **individuals** who bear the risk of

developing CBZ-induced SJS/TEN **in an entire population**. Accordingly, the allele needs to be present at a relatively high frequency in that population so that it can be detected. As set forth below, the cited references merely suggest that HLA-B*1502 may not be a good **prediction marker** to identify risk-bearing individuals in Caucasian population due to its low frequency in that population. In fact, none of the cited references discredits using HLA-B*1502 as a **risk indicator** for patients developing CBZ-induced SJS/TEN, as required by claim 1.

Lonjou reports that among 12 CBZ-induced SJS/TEN patients (4 Asians and 8 Caucasians), none of the 8 Caucasian patients has this allele. See Abstract. Accordingly, it concludes that "HLA-B*1502 is not a useful prediction marker of CBZ related SJS/TEN **in the European population**." See page 3, left column, second paragraph; emphasis added. On the other hand, it is completely silent on whether a Caucasian patient carrying HLA-B*1502 would be at risk for CBZ-induced SJS/TEN, as required by claim 1.

Alfirevic reports that (1) none of 56 CBZ-sensitive Caucasian patients (including 2 SJS/TEN patients) was positive for HLA-B*1502, and (2) none of 43 CBZ-tolerant Caucasian patients carry this HLA allele. See Abstract. It thus concludes that HLA-B*1502 is not a marker for CBZ-induced SJS/TEN **in patients of all ethnicities**. Similar to Lonjou, Alfirevic only addresses whether HLA-B*1502 could be used as a prediction marker in an ethnic group in which the allele is present at a very low frequency. Again, it does not contradict Applicants' position that a patient (in any ethnic group) carrying HLA-B*1502 is at risk for CBZ-induced SJS/TEN.

Relying on the teachings in Lonjou et al. and Alfirevic, i.e., Caucasian SJS/TEN patients being examined do not carry HLA-B*1502, the Examiner concludes that this HLA allele is not associated with the drug reaction in Caucasians. See the Office Action, page 12, second paragraph. Applicants would like to point out that HLA-B*1502 being a risk indicator of CBZ-induced SJS/TEN, as required by claim 1, does not compel the conclusion that it is the only risk indicator of this drug reaction so that every CBZ-induced SJS/TEN patient carries it. In other words, it is likely that Caucasian CBZ-incuded SJS/TEN patients carry risk indicators other than HLA-B*1502. Accordingly, the fact that some CBZ-induced SJS/TEN patients do not carry HLA-B*1502 does not contradict the conclusion that HLA-B*1502 is a risk indicator of this drug reaction, i.e., a patient carrying this HLA allele is at risk for CBZ-induced SJS/TEN.

Hung et al. teaches that "[a]n allele associated with a particular phenotype or disease may be present in different frequencies **in different ethnic groups**." See page 233, left column; emphasis added. Accordingly, when the frequency of an allele is low in a population, its association with a certain disease would be difficult to detect in that population. Indeed, according to this reference, "[t]he allele frequency of HLA-B*1502 is approximately **4-7% in Southern Han Chinese**, but only **0-0.1% in Caucasians**." See page 233, left column, last paragraph through right column, first paragraph; emphases added. This information explains the difficulty in detecting the association between HLA-B*1502 and CBZ-induced SJS/TEN in Caucasians, which is not contradictory to the teachings of the present application, i.e., a patient carrying HLA-B*1502 bears the risk of developing CBZ-induced SJS/TEN.

As a matter of fact, Hung et al. actually supports the position that HLA-B*1502 can be used as a risk indicator of CBZ-induced SJS/TEN in any patient. It states that "the HLA-B*1502 allele is quite common in **Malaysia** (approximately **8.4%** frequency), and CBZ is reported to be the major offending drug for SJS/TEN in that population (**35.7% of SJS/TEN patients**)." See page 233, left column, last paragraph through right column, first paragraph, emphases added. It further reports **low incidence** of CBZ-induced SJS/TEN in **Caucasians**, who have a low allele frequency of HLA-B*1502 (i.e., **0-0.1%**). See page 233, left column, last paragraph through right column, first paragraph. Based on the above information, Hung et al. proceeds to conclude that the HLA-B*1502 allele frequency positively correlates with the prevalence of CBZ-induced SJS/TEN in different populations. See page 233, right column, lines. 8-16. In other words, HLA-B*1502 highly correlates with CBZ-induced SJS/TEN, and could be used as a risk indicator of the drug reaction in any patient.

In sum, none of the three references relied on by the Examiner suggests that HLA-B*1502 is not a risk indicator of CBZ-induced SJS/TEN in a Caucasian patient, i.e., a Caucasian patient carrying this HLA allele is not at risk for SJS/TEN. Absent such suggestion, these references cannot serve as reasonable bases to challenge the enablement of claim 1 in its entire scope, i.e., HLA-B*1502 can be used to assess the risk of developing CBZ-induced SJS/TEN in a patient, regardless of the patient's ethnic background.

In view of the foregoing remarks, Applicants submit that it is improper to reject claim 1 for lack of enablement. Nor is it proper to reject claims 8-12, all of which depend from claim 1. For the same reasons set forth above, it is also improper to reject claims 20 and 22-25 for lack of enablement.

III

Referring to claim 11, the Examiner alleges that "[i]t is unpredictable as to whether or not the presence of any equivalent genetic marker is useful for determining the presence of the HLA-B*1502 allele or for the assessment of risk of drug adverse reaction." See the Office Action, page 8, second paragraph. Applicants disagree.

The Specification defines an "equivalent genetic marker" of an allele of interest as "a genetic marker that is linked to the allele of interest." See page 8, lines 19-20. It further states that "[t]he useful equivalent genetic markers in the present invention display a linkage disequilibrium with the allele of interest." See page 8, lines 20-21. Based on these teachings, one skilled person in the art would know that the presence of an equivalent genetic marker of HLA-B*1502 indicates the presence of this HLA allele. Thus, he or she would recognize that any equivalent genetic marker, as recited in claim 11, could be useful for determining the presence of HLA-B*1502 and thus for assessing the risk of developing CBZ-induced SJS/TEN.

For the above reasons, Applicants respectfully request that the Examiner withdraw this ground for rejecting claim 11.

IV

Finally, referring to claim 12, the Examiner asserts that "there is no statistical analysis of the significance of the association of HLA-Cw*0801 with carbamazepine-induced SJS/TEN, nor any analysis of the linkage of HLA-B*1502 with HLA-Cw*0801." See the Office Action, page 8, second paragraph. Applicants disagree.

It is well known in the art that HLA-B*1502 has a strong linkage disequilibrium with HLA-Cw*0801. See Romphruk et al., *European J. Immunogenet.*, 30:153-158 (2003), page 4, Table 4 and right column, second paragraph; and Romphruk et al., *Tissue Antigens*, 58:83-89 (2001), page 86, Table 4, and page 87, left column, first paragraph. (copies submitted herewith as Exhibits 1 and 2). Further, the Specification teaches that 38 of 42 carbamazepine-induced

SJS/TEN patients (HLA-B*1502 carriers) have HLA-Cw*0801 allele. In other words, as many as 90% (38/42) of the SJS/TEN patients carry HLA-Cw*0801. As the association between HLA-Cw*0801 and CBZ-induced SJS/TEN (90%) is clearly significant, one skilled person in the art would readily recognize that HLA-Cw*0801, an allele co-present with HLA-B*1502, is an indicator of the risk of developing CBZ-induced SJS/TEN in a patient. Indeed, this conclusion is supported by a post-filing reference, Hung et al., which shows that 56 out of 60 SJS/TEN patients carry HLA-Cw*0801. See Hung et al., Pharmacogenetics and Genomics, 16(4):297-306 (2006) (copy submitted herewith as Exhibit 3).

For the reasons set forth above, Applicants submit that the Specification is enabling for claim 12, which recites HLA-Cw*0801. It is thus respectfully requested that the Examiner withdraw this lack-of-enablement rejection.

New Claim

New claim 26 covers a method of assessing the risk of developing CBZ-induced SJS/TEN in a Mongoloid or a descendent of a Mongoloid. The Examiner acknowledges that the Specification is enabling for assessing the risk in Taiwanese patients. See the Office Action, page 3, third paragraph. As all Taiwanese are Mongoloid, this claim, limited to Mongoloid patients, is clearly enabled.

CONCLUSION

In view of the above remarks, it is submitted that claims 1, 8-12, 20, and 22-26 are enabled. Allowance of these claims is respectfully solicited.

Please apply charges or credits, if any, to deposit account 06-1050.

Respectfully submitted,

Date: 3/19/2007

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HLA-B*15 subtypes in the population of north-eastern Thailand

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Summary

The HLA-B*15 group is the most polymorphic HLA-B allele and so has several subtypes. These subtypes have not been defined in the population of north-eastern Thailand (NET). In a previous study, using polymerase chain reaction–sequence-specific primers (PCR-SSP), subtypes were categorized into four groups, namely: group I: HLA-B*15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27, 32, 33, 34 and 35); group II: HLA-B*15 (02, 08, 11, 15, 28 and 30); group III: HLA-B*1503/4802; group IV: HLA-B*1521. Groups I and II occurred frequently (allele frequency = 8.0 and 2.5%), and thus we optimized the polymerase chain reaction–single-stranded conformation polymorphism (PCR-SSCP) method to identify HLA-B*15 subtypes of groups I and II. Eighty samples of DNA carrying HLA-B*15 from 300 healthy unrelated individuals were tested. B*1502 (52.5%) and B*1525 (13.8%) were the most common subtypes found in NET. They also showed strong linkage disequilibrium with HLA-Cw and heterogeneity of HLA-A, DR, DQ haplotypes. Although limited conclusions can be drawn from this study because of the small number of DNA references used, the baseline data will be useful in the selection of common HLA-B*15 alleles when subtyping for unrelated donor transplantations.

Introduction

Human leukocyte antigen (HLA) B15 represents a large, genetically diverse group of HLA-B alleles. It was first described and its heterogeneity recognized at the Fourth International Histocompatibility Workshop in 1970 (4th IHW) (Albert *et al.*, 1970; Thorsby & Kissmeyer-Nielsen, 1970; Thorsby *et al.*, 1970). At the 9th IHW, the heterogeneity of HLA-B15 was summarized and discussed by Chandanayingyong *et al.* (1984) and Cambon-Thomsen *et al.* (1984). At the 10th IHW, seven serological subtypes of B15 were identified and five were recognized by the World Health Organization (WHO) Nomenclature Committee as distinct specificities: B62, B63, B75, B76 and B77 (Albert *et al.*, 1989). Many new HLA-B15 related alleles have since been discovered in different ethnic groups throughout the world, especially among South-east Asians (Alonso *et al.*, 1983; Cambon-Thomsen *et al.*, 1984; Albert *et al.*, 1989; Parham *et al.*, 1989; Pohla *et al.*, 1989; Nisperos *et al.*, 1991; Choo *et al.*, 1991, 1993; Little & Parham, 1991; Belich *et al.*, 1992; Santamaria *et al.*, 1993; Hildebrand *et al.*, 1994; Wang *et al.*, 1997). The correlations of HLA-B15 molecular structures with serological specificities and antigenic properties have been discussed (Pohla *et al.*, 1989; Hildebrand *et al.*, 1994), but the serological assignments of B*15 alleles to B62, B63, B75, B76 and B77 are complicated by cross-reactivities among the antigens encoded by these alleles (Hildebrand *et al.*, 1994; Lin *et al.*, 1996; Steiner *et al.*, 1997; Marsh *et al.*, 2001). Although the heterogeneity of HLA-B15 antigens has been described in both serological and molecular analyses, the serological complexities of this group and the corresponding molecular structures are still unclear.

In the Thai population, HLA-B15 antigens have been investigated in distinct ethnic groups such as the population of central Thailand (Bangkok) (Chandanayingyong *et al.*, 1997), Dai Lue (Chandanayingyong *et al.*, 1997), the Thai and Thai-Chinese population (Imanishi *et al.*, 1991), the population of northern Thailand (Fongsatikul *et al.*, 1997) and that of southern Thailand (Chiewsilp *et al.*, 1997). The most common serologically defined antigens were B75 and B62.

We have studied the distribution of HLA-B*15 alleles in 300 samples from the population of north-eastern Thailand (NET) based on the 12th IHW PCR-amplification refractory mutation system (ARMS). Six groups of B*15 could be defined by this protocol. Four groups of B*15 were identified in this population: B*15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27 and 32–35) [allele frequency (AF) = 3.67%], B*15 (02, 08, 11, 15, 28 and 30) (AF = 8.67%), B*1503/4802 (AF = 0.5%), and B*1521 (AF = 0.5%) (Romphruk *et al.*, manuscript in preparation). Each group was carried by different HLA class I and class II haplotypes. In spite of these differences, the biological significance of the genetic heterogeneity of HLA-B15

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requires to be elucidated. Before this information can be collated, the subtypes of HLA-B15 need to be identified. Thus, our aim was to identify HLA-B*15 subtypes in NET by PCR–single-stranded conformation polymorphism (PCR-SSCP).

Materials and methods

Samples

A total of 80 HLA-B15 positive samples were obtained from 300 unrelated healthy individuals from north-eastern Thailand (Romphruk *et al.*, manuscript in preparation). All of these samples were typed for HLA-B*15 alleles using the 12th IHW PCR-ARMS protocol. Four groups were differentiated, but only two were studied by PCR-SSCP, namely: group I (n = 22), B*15 (01, .04–07, 12, 14, 19, 20, 24, 25, 26N, 27 and 32–35) and group II (n = 52), B*15 (02, 08, 11, 15, 28 and 30). The DNA references for B*15 alleles (namely B*1501, 1502, 1503, 1505, 1507, 1508, 1510, 1511, 1512, 1513, 1516, 1518, 1520, 1524, 1525, 1527, 1528 and 1532) were provided by the Japanese Red Cross Central Blood Center, Tokyo, Japan and the Australian Red Cross Blood Service, Melbourne, Australia.

Primers

A two-step PCR was performed (Fig. 1) using the same nucleotide primer sequence as Lin *et al.* (1996). In the first step, the HLA-B*15 gene fragment was amplified from genomic DNA by PCR using the group-specific primers BEX2-1 and BCT. The amplified fragment was about 742 bp in length encompassing exon 2, intron 2 and most of exon 3. A second PCR was then performed separately to amplify exon 2 (215 bp) and exon 3 (207 bp) using primer pairs BEX2-1/BEX2-FC and BEX3-1/BCT, respectively.

PCR amplifications

PCR amplifications were performed in a 10-µl reaction mixture. In the first PCR, the mixture consisted of 100 ng of genomic DNA as template, 0.5 µM of each primer, 200 µM of each dNTP, 50 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 1.0 mM $MgCl_2$ and 0.5 units of Taq DNA polymerase (Promega, Madison, WI or MHC Research Group, Khon Kaen University (Leelayuwat *et al.*, 1997)). After preheating at 94 °C for 2 min, 35 PCR cycles were performed, followed by final extension at 72 °C for 10 min. Each amplification cycle consisted of 1 min each of denaturing (94 °C), annealing (54 °C) and extension (72 °C). The PCR was carried out in a Gene Amp PCR system 9600 (Perkin Elmer Corporation, Norwalk, CT).

The reaction mixture for the second PCR was the same as that for the first PCR, except that we used 1 µl of the first PCR product as the template, 0.8 mM $MgCl_2$ for exon 2 and 1.2 mM $MgCl_2$ for exon 3. The amplification was performed for 20 cycles, after preheating to 94 °C for 2 min and final extension of 10 min at 72 °C. Each amplification cycle consisted of denaturing (94 °C) for 30 s, annealing (58 °C) for 30 s, and extension (72 °C) for 1 min. The presence or absence of PCR product was determined by gel electrophoresis. Five microlitres of PCR product was run on a 1% agarose gel containing 0.5 µg ml^{-1} of ethidium bromide and visualized under UV illumination.

SSCP fragment analysis

The exon 2 and 3 fragments amplified by the second PCR were analysed separately using the SSCP method (Lin *et al.*, 1996; Maruya *et al.*, 1996). Electrophoresis was carried out in a Miniprotein II polyacrylamide gel (Mini-PROTEAN II, Biorad, Hercules, CA) (Table 1). The single-stranded DNA fragments separated in the gel were detected by silver staining.

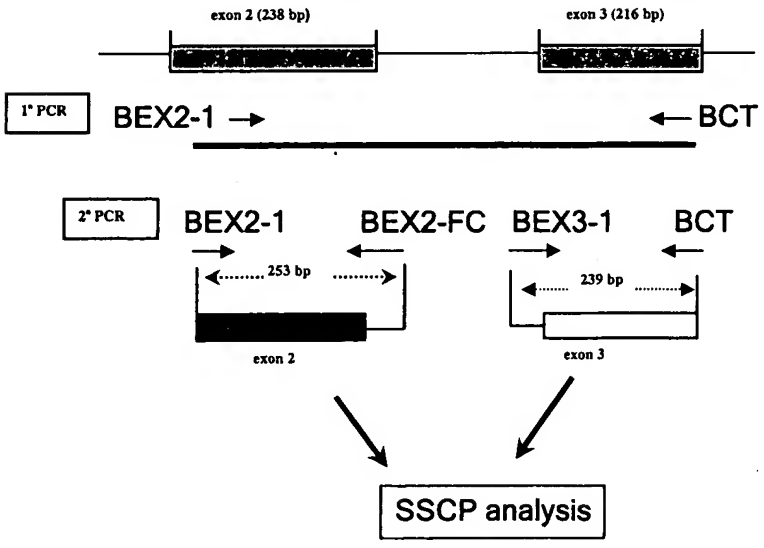

Figure 1. The strategy of SSCP fragment analysis of exons 2 and 3 in HLA-B*15 alleles. Primers BEX2-1 and BCT are group-specific primers. BEX2-1/BEX2-FC and BEX3-1/BCT amplified the exon 2 and exon 3 seperately.

Table 1. SSCP conditions for groups I and II of the B*15 alleles

Group		Electrophoretic temperature (°C)	Voltage (v)	Time (min)	Gel concentration	Acrylamide:bis
Group I	exon 2	4	200	150	10%	39 : 1
B15* (01, 04–07, 12, 14, 19, 20, 25, 26N, 27, 32–35)	exon 3	4	150	150	7%	50 : 1
Group II	exon 2	4	150	150	10%	50 : 1
B*15 (02, 08, 11, 15, 28, 30)	exon 3	4	150	60	7%	50 : 1

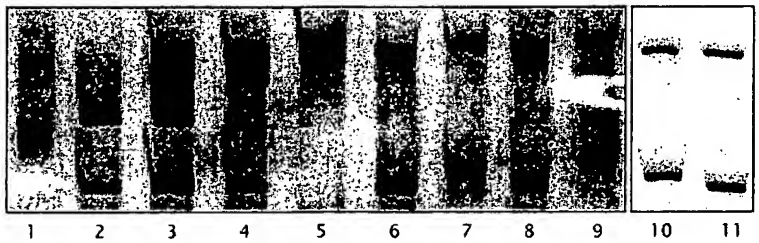

Figure 2. PCR-SSCP analysis of HLA-B15 group I (1501, 1505, 1507, 1512, 1520, 1524, 1525, 1527, 1532). Lanes 1 (B*1525), 2 (B*1532), 3 (B*1507), 4 (B* 1520), 5 (B* 1512), 6 (B* 1524), 7 (B*1527), 8 (B* 1501) and 9 (B* 1505) were distinguished using exon 3 fragment analysis. Lanes 10 (B*1501) and 11 (B*1524) by exon 2.

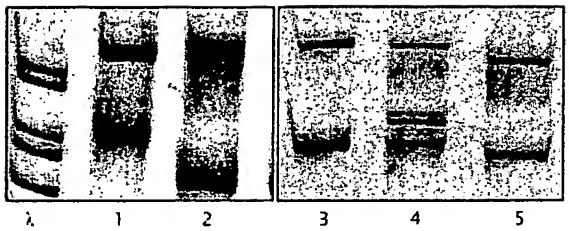

Figure 3. PCR-SSCP analysis of HLA-B15 Group II (1502, 1508, 1511, 1528). Lanes 1 (B*1502) and 2 (B*1508/1511/1528) were done using exon 3 fragments analysis. Lanes 3 (B*1508), 4(B*1511) and 5 (B*1528) by exon 2.

Reproducibility of the test

To ensure reproducibility, the DNA references were tested within and between runs for both PCR amplification and SSCP fragment analysis.

Results

SSCP fragment analysis

Most of alleles in group I had nucleotide sequence differences in exon 3, so analysis was used to discriminate alleles B*1505, 07, 12, 20, 25, 27 and 32. The other two alleles, B*1501 and B*1524, were defined by the exon 2 SSCP analysis (Fig. 2). In group II, exon 3 SSCP analysis discriminated B*1502 from B*1508, 1511 and 1528 and exon 2 discriminated B*1508, B*1511 and B*1528 (Fig. 3).

Reproducibility of the test

The SSCP analysis of DNA references for exon 2 for both groups I and II showed identical banding patterns 'within' and 'between' runs. The exon 3 analysis showed slight differences in banding patterns on the 'between run' assay, but these alleles were easily defined. Thus, the DNA references were included in every SSCP analysis.

B*15 alleles in NET

Eight different patterns of HLA-B*15 alleles (groups I and II) were detected in NET (Table 2). The common alleles were B*1502 (52.5%), B*1525 (13.8%) and B*1528 (5.0%). Three samples of group I [B*15 (01, 07)?] could not be clearly defined because extra bands from the other HLA-B allele in these samples could not be excluded. One of the 22 samples from group I and two of the 52 in group II were undefined because they gave different banding patterns from the DNA references. They might have been B*15 (04, 06, 14, 19 or 33–35) or B*15 (15 or 30) for which we have no DNA references. Five samples from group I and three from group II could not be analysed because DNA samples were not available. The comparison of B*15 alleles in NET and other populations is presented in Table 3.

Haplotype analysis

Table 4 summarizes the possible association of HLA-B*15 alleles with other HLA alleles. The data for other loci are from a previous study (Romphruk *et al.*, manuscript in preparation). Only four haplotypes in Table 4 could be confirmed by family segregation analysis. Most HLA-B*1502 haplotypes (92.85%) carried HLA-Cw*08 but carried diverse alleles at HLA-A, -DR and -DQ. All HLA-B*1525 and B*1521 haplotypes carried HLA-Cw*04 but carried diverse alleles at HLA-A, -DR and -DQ. In addition, associations of B*1528 and B*1532 with Cw*07 (01–03) were observed.

Table 2. B*15 alleles in HLA-B15 positive samples (n = 80) from 300 healthy individuals from north-eastern Thailand

B*15[a]	B*15 alleles	n (%) (n = 80)
Group I	1501	—
	1505	—
	1507	—
	1512	—
	1520	—
	1524	—
	1525	11 (13.8%)
	1527	—
	1532	2 (2.5%)
	15 (01, 07)?	3 (3.7%)
	15 (04, 06, 14, 19, 33, 34, 35)	1 (1.3%)
	Not done	5 (6.2%)
Group II	1502	42 (52.5%)
	1508	—
	1511	1 (1.3%)
	1528	4 (5.0%)
	15 (15, 30)?	2 (2.5%)
	Not done	3 (3.7%)
Group III	1503/4802	3 (3.7%)
Group IV	1521	3 (3.7%)
Group V	15 (13, 16, 17)	0
Group VI	15 (09, 10, 18, 23)	0

[a] The B15 groups were defined according to the 12th IHW PCR-ARMS.

Table 3. Percentage of HLA-B*15 subtypes in various populations

B*15	NET (n = 80)	Vietnamese[e] (n = 43)	Caucasian[e] (n = 70)	Ni-Nanuatu[e] (n = 37)	Bubi[e] (n = 30)	Koreans[f] (n = 237)	Japanese[g] (n = 53)
1501	3.75[a]	20.9	85.7[d]	5.4		35.0[d]	30.2[d]
1502	52.5	55.8				1.5[d]	0.9[d]
1503	3.7[b]				53.3[d]		ND
1506	ND			89.19[d]			ND
1507			1.4			4.6	4.7
1509			1.4				ND
1510					40.0[d]		ND
1511	1.3					7.6[d]	2.8
1512		14.0[d]					ND
1515	2.5[c]					ND	ND
1516					10.0		ND
1517			5.7			0.002	ND
1518	ND		7.1			3.0	10.4
1521	3.7	2.3		8.1			ND
1525	25.6	14.0				0.002[d]	ND
1527		2.3				1.5	0.9
1528	5.0						ND
1532	2.5					ND	ND
1538	ND	ND	ND	ND	ND	0.004	ND

Blank = not found. ND = not done/no data. [a] B15 (01 or 07); [b] B1503/4802; [c] B15 (15 or 30); [d] significant difference compared to NET (corrected $P < 0.05$); [e] data from Barnardo *et al.* (1998); [f] data from Lee *et al.* (2000); [g] data from Saito *et al.* (2000).

Discussion

HLA-B*15 is a diverse group of alleles of the HLA-B locus and is found throughout the world, suggesting that its lineage is as old as *Homo sapiens* (Hildebrand *et al.*, 1994). By DNA sequencing of exons 2 and 3, 68 B*15 alleles were defined (Marsh *et al.*, 2001). B*15 alleles associated with specific ethnic groups have been described, including B*1518 in Asian Indians and B*1521 in Australian Aborigines (Lienert *et al.*, 1995).

We used PCR-SSCP to define the B*15 allele in NET. This technique depends on the conformation of single-stranded DNA to produce the different banding patterns, so DNA references are needed for comparisons. However, the references themselves varied under different conditions. Unfortunately, we did not have all the DNA references,

Table 4. Possible associations of B*15 alleles with other HLA loci

B*	A	Cw*	DRB1*	DQB1*	%
1502		08			92.85 (39/42)
	11	08	1202	0301	26.19 (11/42)
	24	08	1501	0601	26.19 (11/42)
	11	08	1502	0501	4.76 (2/42)
1525		04			100 (11/11)
		04	1106	0301	27.27 (3/11)
		04	1502	0501	18.18 (2/11)
		04	1502	0502	18.18 (2/11)
1521	34/66	04			100 (3/3)
1528		07(01–03)			50 (2/4)
1532		07(01–03)			100 (2/2)

so only nine of the 17 alleles for group I [B*15 (01, 05, 07, 12, 20, 24, 25, 27 and 32)] and four of the six for group II [B*15 (02, 08, 11 and 28)] were included in our study. Specific primers can amplify other alleles at the B locus, such as B*4601, B*5801, B*38, B*39 and B*35 (Lin *et al.*, 1996), so, in addition to the two alleles per locus in individual samples, the SSCP pattern revealed other bands which were not from B*15 alleles. Thus, including other known alleles present in the sample in the SSCP fragment analysis will help to clarify the B*15 bands.

HLA-B15 allelic types and their frequencies have previously been shown to vary in different populations (Marsh *et al.*, 2001; Lee *et al.*, 2000). Frequencies of some of the most common B*15 alleles have been determined: B*1502 in NET (52.5%) and Vietnamese (55.8%); B*1501 in Caucasians (85.7%), Koreans (35.0%), and Japanese (30.2%); B1506 in Ni-Vanuatu (89.2%); B*1503 and B*1510 in Bubi (53.3 and 40.0%). The distribution of the B*15 alleles in NET is similar to that in the Vietnamese, except for B*1512. Compared to other Thai groups (Thais and Thais-Chinese, Thais from Central Thailand and Dai Lue), B*75 (encoded by B*1502, 1508, 1511 and 1521), B62 (encoded by B*1501, 1504–1507, 1515, 1520, 1524, 1525, 1527, 1530, 1532, 1545, and 1548) and B77 (encoded by B*1513) were found more frequently (Chandanayingyong *et al.*, 1997; Imanishi *et al.*, 1991). It is possible that B75 was encoded by B*1502 and that most of B62 was encoded by B*1525 in NET. In contrast, B*1501 was the predominant allele for the B62 antigen in Koreans (166 in 198 samples) but B*1525 was rare (1 in 198 samples) (Lee *et al.*, 2000). B*1513 was not found in the present study, indicating that this allele is rare in north-eastern Thailand. In a separate study, we found this allele in only one in 240 cases of cholangiocarcinoma in patients from north-eastern Thailand (Romphruk *et al.*, manuscript in preparation). This confirms our observation that the distribution of MHC alleles in the population of north-eastern Thailand is different from that in the population of Central Thailand, which shows a high degree of Thai-Chinese admixture (Romphruk *et al.*, 1999).

HLA-B15, combined with B4601, is the most common (22–30%) HLA-B allele in south-east Asians, including the Chinese. Hildebrand *et al.* (1994) and Lin *et al.* (1996) proposed a phylogenetic tree for 26 HLA-B15 alleles and a network of structure relationships among these alleles. B4601 may have evolved from B*1501 — the putative original B*15 allele (Zemmour *et al.*, 1992; Hildebrand *et al.*, 1994; Lin *et al.*, 1996;). B*4601 and B*1501 differ in the short codon position 66–76, which may be the result of segmental conversion between B*1501 and Cw*01. Thus, HLA-B*4601 in Asians may have originated from B*1501 via gene conversion. This hypothesis, combined with selective pressure, may explain the low frequency of B*1501 and the high frequency of B*4601 in these populations.

The possible associations of B*15 with other loci are presented in Table 4. The possible B*15–Cw* association showed several different patterns. B*1502 was strongly associated with Cw*08, B*1525 with Cw*04, and B*1528 and B*1532 with Cw*07 (01–03). B*1502 and B*1525 were more diverse in their associations with HLA-A, Cw, DR and DQ than other B*15 alleles. This suggests that these two alleles may be relatively old alleles in the Thai population, similar to B*1501 and B*1518 in Koreans. The diversity of haplotypes is likely to be the result of recombination between HLA-B and other loci during the long period of haplotype formation in the population (Lee *et al.*, 2000). The strong linkage disequilibrium between B*15 alleles and HLA-Cw will be useful in discriminating the B15 subgroups, especially where B*15 subtyping is not available.

In conclusion, we defined B*15 alleles and the heterogeneity of haplotypes carrying HLA-B15 in NET. High-resolution typing will be necessary to select unrelated donors for organ and bone marrow transplantation. The data obtained in this and similar studies will facilitate the selection of common B*15 alleles for subtyping in these populations.

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References

Albert, E.D., Mickey, M.R., McNicholas, A.C. & Terasaki, P.I. (1970) Seven new HLA-A specificities and their distribution in three races. In: *Histocompatibility Testing* (ed. by P. I. Terasaki), p. 221. Munksgaard, Copenhagen.

Albert, E.D., Chandanayingyong, D., Thomsen, J.S., Hammond, M.G., Naito, S. & Sierp, G.M. (1989) Antigen Society #9 Report (Bw46 and the subgroups of B15). In: *Immunobiology of HLA, Volume I* (ed. by B. Dupont), p. 153. Springer-Verlag, New York.

Alonso, A., Ollier, W., Doyle, P., Williams, E. & Festenstein, H. (1983) Family investigation demonstrating the association of HLA-Bw62 with HLA-Bw4. *Tissue Antigens*, 22, 32.

Barnardo, M.C., Welsh, K.I., Vilches, C., Maitland, K. & Bunce, M. (1998) Allele-specific HLA-B*15 typing by PCR-SSP and its application to four distinct ethnic populations. *Tissue Antigens*, 51, 293.

Belich, M.P., Madrigal, J.A., Hildebrand, W.H., Zemmour, J., Williams, R.C., Luz, R., Petzl-Erler, M.L. & Parham, P. (1992) Unusual HLA-B alleles in two tribes of Brazilian Indians. *Nature*, 357, 326.

Cambon-Thomsen, D., Chandanayingyong, D., Thomsen, M. & Hammond, M.G. (1984) Antigen Report: HLA-Bw4-associated variants of B15. In: *Histocompatibility Testing* (ed. by E. D. Albert), p. 171. Springer-Verlag, Heidelberg.

Chandanayingyong, D., Cambon-Thomsen, A. & Hammond, M.G. (1984) Antigen Report: HLA-Bw62 and other Bw6-associated variants of B15. In: *Histocompatibility Testing* (ed. by E. D. Albert), p. 169. Springer-Verlag, Heidelberg.

Chandanayingyong, D., Stephens, H.A., Klaythong, R., Sirikong, M., Udee, S., Longta, P., Chantangpol, R., Bejrachandra, S. & Rungruang, E. (1997) HLA-A-B-DRB1-DQA1, and -DQB1 polymorphism in Thais. *Human Immunology*, 53, 174.

Chiewsilp, P., Mongkolsuk, T., Sujirachato, K., Junpong, S., Rattanasombat, K. & Uden, C. (1997) HLA in southern Thai-Muslims. *Journal of the Medical Association of Thailand*, 80, 30.

Choo, S.Y., Abramov, V. & Hansen, J.A. (1991) Molecular heterogeneity in the HLA-Bw62 antigen. In: *HLA 1991, Volume I* (ed. by K. Tsuji, M. Aizawa & T. Sasazuki), p. 833. Oxford University Press, Oxford.

Choo, S.Y., Fan, L.A. & Hansen, J.A. (1993) Allelic variations clustered in the antigen binding sites of HLA-Bw62 molecules. *Immunogenetics*, 37, 108.

Fongsatikul, L., Nantachit, N., Kamtorn, N. & Leetrakool, N. (1997) HLA gene frequencies of northern Thais. *Journal of the Medical Association of Thailand*, 80, 38.

Hildebrand, W.H., Domena, J.D., Shen, S.Y., Lau, M., Terasaki, P.I., Bunce, M., Marsh, S.G., Guttridge, M.G., Bias, W.B. & Parham, P. (1994) HLA-B15: a widespread and diverse family of HLA-B alleles. *Tissue Antigens*, 43, 209.

Imanishi, T., Akaza, T., Kimura, A., Tokunaga, K., & Gojobori, T. W15.1 allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: *HLA 1991, Volume I* (ed. by K. Tsuji, M. Aizawa & T. Sasazuki), p. 1071. Oxford University Press, Oxford.

Lee, K.W., Jeon, H. & Park, J.Y. (2000) HLA-B*15 diversity in the Korean population. *Tissue Antigens*, 56, 428.

Leelayuwat, C., Srisuk, T., Paechaiyaphum, R., Limpaiboon, T., Romphruk, A. & Romphruk, A.V. (1997) Production and evaluation of Tag DNA polymerase. *Journal of the Medical Association of Thailand*, 80, S129.

Lienert, K., McCluskey, J., Bennett, G., Fowler, C. & Russ, G. (1995) HLA class I variation in Australian aborigines: characterization of allele B*1521. *Tissue Antigens*, 4, 12.

Lin, L., Tokunaga, K., Tanaka, H., Nakajima, F., Imanishi, T., Kashiwase, K., Bannai, M., Mizuno, S., Akaza, T. & Tadokoro, K. *et al.* (1996) Further molecular diversity in the HLA-B15 group. *Tissue Antigens*, 47, 265.

Little, A.M. & Parham, P. (1991) The HLA-Bw75 subtype of B15: molecular characterization and comparison with crossreacting antigens. *Tissue Antigens*, 38, 186.

Marsh, S.G., Bodmer, J.G., Albert, E.D., Bodmer, W.F., Bontrop, R.E., Dupont, B., Erlich, H.A., Hansen, J.A., Mach, B. & Mayr, W.R. *et al.* (2001) Nomenclature for factors of the HLA system, 2000. *Tissue Antigens*, 57, 236.

Maruya, E., Saji, H. & Yokoyama, S. (1996) PCR-LIS-SSCP (Low ionic strength single-stranded conformation polymorphism) — a simple method for high-resolution allele typing of HLA-DRB1-DQB1, and -DPB1. *Genome Research*, 6, 51.

Nisperos, B.B., Chung, K.H., Hansen, J.A., Chan, S.H., Stewart, D., Pazderka, F., Tada, M., Chandanayingyong, D., Fan, L. & Berg-Loonen, E.V.D. *et al.* (1991) Antigen Society Reports no. 108: HLA-B15-B62-B63-B75-B76-B77-B46. In: *HLA 1991, Volume I* (ed. by K. Tsuji, M. Aizawa & T. Sasazuki), p. 310. Oxford University Press, Oxford.

Parham, P., Lawlo, R.F.A., Salter, R.D., Lomen, C.E. & Ennis, P.F. (1989) HLA-A, B, C: patterns of polymorphism in peptide binding proteins. In: *Immunobiology of HLA, Volume II* (ed. by B. Dupont), p. 10. Springer-Verlag, New York.

Pohla, H., Kuon, W., Tabaczewski, P., Doerner, C. & Weiss, E.H. (1989) Allelic variation in HLA-B and HLA-C sequences and the evolution of the HLA-B alleles. *Immunogenetics*, 29, 297.

Romphruk, A.V., Puapairoj, C., Romphruk, A., Barasrux, S., Urwijitaroon, Y. & Leelayuwat, C. (1999) The distribution of HLA-DRB1/DQB1 alleles and haplotypes in the north-eastern Thai population: indicative of a distinct Thai population with Chinese admixtures in the central Thais. *European Journal of Immunogenetics*, 26, 129.

Saito, S., Ota, S., Yamada, E., Inoko, H. & Ota, M. (2000) Allele frequencies and haplotypic associations defined by allelic DNA typing at HLA class I and class II loci in the Japanese population. *Tissue Antigens*, 56, 522.

Santamaria, P., Lindstrom, A.L., Boyce-Jacino, M.T., Myster, S.H., Barbosa, J.J., Faras, A.J. & Rich, S.S. (1993) HLA class I sequence-based typing. *Human Immunology*, 37, 39.

Steiner, N., Ng, J., Bush, J., Hartzman, R.J., Johnston-Dow, L. & Hurley, C.K. (1997) HLA-B alleles associated with the B15 serologically defined antigens. *Human Immunology*, 56, 84.

Thorsby, E. & Kissmeyer-Nielsen, F. (1970) New HL-A alleles. Identification by planned immunization. *Vox Sanguinis*, 18, 134.

Thorsby, E., Kissmeyer-Neilson, F. & Svejgaard, A. (1970) New alleles of the HLA system: serological and genetic studies. In: *Histocompatibility Testing* (ed. by P. I. Terasaki), p. 137. Munksgaard, Copenhagen.

Wang, J., Fernandez-Vina, M.A., Lazaro, A.M., Shumway, W., LeFor, W. & Stastny, P. (1997) New alleles of the HLA-B15 family. *Human Immunology*, 55, 184.

Zemmour, J., Gumperz, J.E., Hildebrand, W.H., Ward. F.E., Marsh, S.G., Williams, R.C. & Parham, P. (1992) The molecular basis for reactivity of anti-Cw1 and anti-Cw3 alloantisera with HLA-B46 haplotypes. *Tissue Antigens*, 39, 249.

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Diversity of MICA (PERB11.1) and HLA haplotypes in Northeastern Thais

Key words:
HLA; MICA; MHC haplotypes; polymorphism; Thais

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Abstract: MICA or PERB11.1 is a polymorphic major histocompatibility complex (MHC) class I-related gene located 46 kb centromeric of the HLA-B gene in the HLA class I region. It is expressed mainly in gut epithelial cells, keratinocytes, endothelial cells, fibroblasts and monocytes, and is upregulated by heat stress. MICA has been found to interact with γδ T cells, αβ CD8$^+$ and natural killer (NK) cells bearing the NKG2D/DAP10 receptor. The MICA gene displays a high degree of polymorphism with at least 54 alleles. In the present study, polymorphic exons 2, 3 and 4 of the MICA gene were analyzed using sequencing based typing (SBT) in 255 unrelated healthy northeastern Thais. Thirteen previously reported MICA alleles were detected. MICA*008, *010, *002 and *019 were highly predominant with the allele frequencies of 21.4%, 18.2%, 17.6% and 15.3%, respectively. Five of these 13 MICA alleles show significantly different frequencies from those of the Japanese and Caucasian populations. Interestingly, MICA052, which is a very rare allele in other populations, was prevalent with the allele frequency of 8.2%, mainly on the HLA haplotype carrying HLA-B*13 in this population. Strong linkage disequilibria were observed between MICA and HLA-B, as similarly observed in other populations, namely MICA*010-B*4601, MICA052-B*13, MICA*002-B*5801, and MICA*019-B*15 (1502, 1508, 1511, 1515, 1528, 1530). A large variety of three-locus (MICA – HLA-B – HLA-Cw) and six-locus (HLA-DQB1 – HLA-DRB1 – MICA – HLA-B – HLA-Cw – HLA-A) haplotypes were recognized in the northeastern Thai population. This is the first report on MICA allelic distribution in Southeast Asian populations. These data will provide the important basis for future analyses on the potential role of the MICA gene in disease susceptibility and transplantation matching in Southeast Asian populations.

The telomeric region of the human major histocompatibility complex (MHC) includes the MHC class I chain-related (MIC) (1) or PERB11 (2) gene family consisting of seven members: MICA (PERB11.1), MICB (PERB11.2), MICC (PERB11.3), MICD (PERB11.4), MICE (PERB11.5), MICF and MICG (3, 4). Of these, only MICA and MICB are expressed, encoding cell surface glycoproteins of 383 amino acids. The MICA gene is located approximately 46 kb centromeric of HLA-B. MICA molecules are composed of three extracellular domains (α1, α2 and α3), a transmembrane segment and a carboxy-terminal cytoplasmic

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tail similar to other HLA class I molecules. However, MICA shares only an average of 21, 19 and 34% amino acid identity in the α1, α2 and α3 extracellular domains, respectively, with classical HLA class I proteins. Further, MICA and HLA class I gene products differ regarding tissue distribution, regulation of expression, association with β_2-microglobulin and immunological function.

MICA has a diverse tissue distribution. It is transcribed in fibroblast, epithelial cell lines, gastrointestinal epithelium, freshly isolated keratinocytes, endothelial cells and monocytes but not in B lymphocyte (5, 6). MICA is also expressed in some lung, breast, kidney, ovary, prostate and colon carcinoma (7). Expression of the gene is not affected by γ-interferon stimulation but is regulated by promoter heat-shock elements and strongly induced upon cell stress, thus MICA may function as an indicator of cell stress (8, 9). MICA is recognized by a subset of γδT cells that predominate in intestinal and other epithelial sites (8, 9), which suggests that MICA facilitates γδT-cell detection of infected, damaged, or otherwise stressed epithelial cells, thus forming part of the innate immune system (10). Recently, MICA has been reported to be a ligand for an activating receptor, NKG2D, which is expressed on most natural killer (NK) cells, CD8+αβT cells and γδT cells (11). This interaction might activate NK-cell and T-cell responses against MICA-bearing tumors.

The MICA gene displays a high degree of genetic polymorphism in exons 2, 3 and 4, amounting to at least 54 alleles (12–14). In contrast to classical class I genes, the polymorphic sites are distributed throughout the domains but outside the peptide-binding groove of the MHC class I molecules (12). They are defined by a total of 40 nucleotide substitutions, 30 of which are nonsynonymous; 5 of 8 in exon 2, 15 of 16 in exon 3 and 10 of 16 in exon 4 (13). Genetic polymorphism was also recognized in the transmembrane region including indels and variable number of the GCT triplet repeats (4). However, information on the MICA diversity in human ethnics is limited, being derived mainly from the Caucasian and Japanese populations. In the present study, we have used sequencing based typing (SBT) to define MICA alleles in the native Northeastern Thai (NET) population. The results were compared with those from the Caucasian and Japanese populations. Linkage disequilibria among MICA and HLA alleles were also investigated to identify representative HLA haplotypes in the NET population.

Material and methods

Genomic DNA

Peripheral blood cells were collected from 255 unrelated healthy Northeastern Thai individuals. All of them were interviewed for their ancestors. Their families are living in the northeast of Thailand for at least two generations. Genomic DNAs were extracted from buffy coat by the salting-out method (15). Their HLA class I (HLA-A, -B and -C) and class II alleles (HLA-DRB1 and -DQB1) were typed by the polymerase chain reaction-sequence-specific primer (PCR-SSP) technique (Romphruk et al., in preparation). Genomic DNA from an HLA homozygous tissue culture cell line (HTLC), BM15 with MICA*004 was employed as a standard DNA in the SBT protocol.

PCR amplification and sequence determination of the MICA gene

A 2.2-kb MICA gene fragment from exon 2 to exon 5 was amplified by a pair of PCR primers, 5MICA and 3MICA (12). PCR conditions using these primers were described previously (16) with some modifications. Namely, PCR was carried out in a 50-μl reaction using 100–200 ng of DNA and 0.5 units AmpliTaq™ DNA polymerase in the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 95°C for 15 min, followed by 35 cycles of 95°C 30 s, 61°C for 1 min, 72°C for 2 min and final elongation at 72°C for 10 min.

Cycle sequencing

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and subjected to sequence determination using the ABI PRISM™ BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase, FS (Applied Biosystems). Each of exons 2, 3 and 4 was separately sequenced on both DNA strands using six sequencing primers as described by Katsuyama et al. (16). The products were purified by Centri-Sep™ spin columns (Princeton Separations, Adelphia, NJ, USA). The reactions were run on the ABI 377 or ABI 310 Sequencing System (Applied Biosystems) and the results were analyzed using the ABI Sequence Analysis and Navigator Softwares. Raw sequencing data were manually inspected for confirmation and alleles were assigned by alignment with known 54 MICA alleles of the extracellular domains (13, 14).

Statistical analysis

Allele frequency (AF) was calculated using the following formula: %AF=sum of each individual allele/2N ×100. Haplotype frequency (HF) and linkage disequilibrium (D) were calculated by the maximum likelihood method assuming the Hardy-Weinberg law. This value indicates an association between the two loci. Significance of two-locus association was assessed by the Chi-square test. The

significance of the distribution of alleles between NET and other populations was tested by *Pc* value (corrected *P*-value) after the Chi-square method.

Results

MICA allele frequencies in Northeastern Thais

The distribution of MICA alleles was determined by sequencing based typing in 255 unrelated healthy Northeastern Thai individuals (NET). Thirteen alleles were recognized in this population. The allele frequencies (AF) of the MICA gene are listed in Table 1. The common alleles were MICA*008 (21.4%), MICA*010 (18.2%), MICA*002 (17.6%) and MICA*019 (15.3%). In contrast, MICA*020 (0.4%) and *016 (0.2%) were infrequent. Most of the "blank" allele cases revealing the frequency of 7.8% possibly represent a MICA homozygote, but some or a significant fraction of them may correspond to a MICA deletion allele linked to HLA-B*4801 in Asian populations (17, 18). In fact, B*4801 was not so frequent, but was recognized with the allele frequency of around 0.5% in NET (Romphruk et al., unpublished data).

Comparison of MICA allele frequencies among NET, Japanese and Caucasians

The allelic distribution of the MICA gene in NET was compared with those of the Japanese (16) and Caucasian (19) populations (Table 2). As a result, it must be most notable that MICA052 (MICA052 has not be officially recognized and so * is not attached between MICA and 052) (14) was considerably prevalent with the frequency of 8.2% in NET, but absent in Japanese or Caucasians. Further, the significantly high frequencies of MICA*010 (the second most frequent allele in NET), MICA*018 and MICA*019 were found in NET as compared to the Japanese and Caucasian populations by the corrected *P*-value test. On the other hand, the frequencies of MICA*008 were significantly decreased in NET as compared to those of both populations, however, MICA*008 was the most frequent allele amongst all the three populations including NET. In addition, the significantly low frequency of MICA*004 and MICA*009 was observed in NET, only when compared to the Japanese population. There was no MICA*001, MICA*007 or MICA051 (MICA051 has not be officially recognized) (14) allele in NET, which was present with the low frequency in the Japanese and Caucasian populations.

Allele frequencies of MICA in 255 Northeastern Thais (NET)

MICA	*n*	% AF
002	90	17.6
004	18	3.5
008	109	21.4
009	12	2.4
010	93	18.2
012	16	3.1
016	1	0.2
017	11	2.2
018	31	6.1
019	78	15.3
020	2	0.4
026	7	1.4
052	42	8.2

Table 1

Comparison of MICA alleles of NET with those of Japanese and Caucasians

	%AF		
MICA	NET *n*=255	Japanese (16)** *n*=130	Caucasians (19)** *n*=242
001	0.0	0.0	3.0*
002	17.6	14.6	13.0
004	3.5	9.2*	6.0
007	0.0	1.2	2.0*
008	21.4	30.8*	55.0*
009	2.4	16.5*	3.0
010	18.2	10.8*	5.0*
011	0.0	0.0	2.0
012	3.1	12.3	2.0
016	0.2	0.0	2.0
017	2.2	0.0	4.0
018	6.1	0.0*	2.0*
019	15.3	3.5*	0.0*
020	0.4	0.0	1.0
026	1.4	0.0	0.0
051	0.0	0.0	2.0*
052	8.2	0.0*	0.0*

* significantly different (Pc<0.05)
** reference no.

Table 2

Linkage disequilibrium and haplotypes between MICA and HLA-B in NET

Linkage disequilibrium parameters (delta values) and haplotype frequencies (HF) between MICA and HLA-B alleles were calculated in NET. Strong associations were observed between these two loci, as previously reported in the Japanese population (16). MICA – HLA-B associations with statistical significance at the *P* level of less than

Significant MICA and HLA-B associations in 255 NET

MICA	HLA-B	*n*	% HF	Delta	Chi-square
002	35	20	3.9	2.3	21.5
002	38	14	2.8	1.7	17.5
002	5801	49	9.6	7.1	94.8
004	44	17	3.3	3.1	224.8
008	27	26	5.1	3.3	27.5
008	4001	33	6.5	4.6	45.5
008	07 (02–07)	13	2.5	1.1	4.7
008	39/6701	21	4.1	2.3	14.4
008	40 (02,04–06)	16	3.1	1.8	16.9
009	51/5201	10	2.0	1.9	99.3
010	4601	75	14.7	11.6	160.6
010	15a	18	3.5	2.4	17.0
012	5401	4	0.8	0.8	60.7
012	55/56	11	2.2	2.0	99.0
017	57	11	2.2	2.1	232.8
018	18	30	5.9	5.1	158.3
019	1521	8	1.6	1.2	16.3
019	15a	13	2.5	1.4	6.9
019	15b	46	9.0	6.5	87.5
026	07 (02–07)	6	1.2	0.9	52.0
052	13	41	8.0	7.6	215.4

15a=15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27, 32–35)
15b=15 (02, 08, 11, 15, 28, 30)

Table 3

0.05 corresponding to a delta-value of equal to or more than 0.8 are listed in Table 3 with 21 representative haplotypes. The most common haplotype was MICA*010-B*4601 (14.7%), followed by MICA*002-B*5801 (9.6%), MICA*019-B*15(02,08,11,15,28,30) (9.0%) and MICA052-B*13 (8.0%). The NET population was characterized by a large variety of associations of MICA with HLA-B, such as MICA*002, MICA*008, MICA*010, MICA*012 and MICA*019 showing linkage disequilibria with multiple HLA-B alleles as shown in Table 3.

Multi-locus haplotypes in NET

Frequencies of three-locus (MICA – HLA-B – HLA-Cw) and six-locus (HLA-DQB1 – HLA-DRB1 – MICA – HLA-B – HLA-Cw – HLA-A) haplotypes were calculated by incorporating the above MICA – HLAB haplotypes into the known HLA class I and class II haplotypes in NET, and those with more than 0.4% (more than two individuals carrying the same haplotype) are listed in Tables 4 and 5, respectively. They revealed the presence of diversed and con-

Possible MICA – B – Cw haplotypes in 255 NET

MICA	HLA-B	HLA-Cw	*n*	%HF
008	07 (02–07)	07 (01–03)	11	2.2
026	07 (02–07)	15 (02,03,05)	5	1.0
052	13	0304	22	4.3
052	13	04	18	3.5
008	13	0602	5	1.0
019	15a	04	9	1.8
010	15a	07 (01–03)	6	1.2
019	15b	04	4	0.8
019	15b	08	36	7.1
010	15b	07 (01–03)	2	0.4
019	1521	04	7	1.4
008	18	07 (01–03)	4	0.8
019	18	07 (01–03)	5	1.0
018	18	0704	28	5.5
018	18	04	2	0.4
008	27	0304	17	3.3
008	27	07 (01–03)	2	0.4
019	27	1202	3	0.6
002	35	04	13	2.6
002	38	07 (01–03)	10	2.0
002	39/6701	07 (01–03)	2	0.4
008	39/6701	07 (01–03)	18	3.5
008	40 (02,04–06)	15 (02,03,05)	5	1.0
008	40 (02,04–06)	0304	2	0.4
008	4001	0303	3	0.6
008	4001	0304	7	1.4
008	4001	04	6	1.2
008	4001	07 (01–03)	14	2.8
004	44	07 (01–03)	16	3.1
010	4601	01	73	14.3
020	5001	0602	2	0.4
019	51/5201	07 (01–03)	2	0.4
002	51/5201	14	6	1.2
009	51/5201	14	5	1.0
009	51/5201	15 (02,03,05)	3	0.6
012	5401	01	4	0.8
012	55/56	01	6	1.2
012	55/56	1202	2	0.4
002	55/56	1203	2	0.4
017	57	0602	11	2.2
002	5801	0302	42	8.2

15a=15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27, 32–35)
15b=15 (02, 08, 11, 15, 28, 30)

Table 4

Possible MICA and 5- locus HLA haplotypes in 255 NET *Table 5*

DQB1	DRB1	MICA	B	Cw	A	*n*	%HF
0501	1001	026	07 (02–07)	15 (02,03,05)	29	4	0.8
0501	1502	052	13	04	11	4	0.8
0502	1602	052	13	0304	11	5	1.0
02	07	008	13	0602	30	5	1.0
0502	1502	010	15a	07 (01–03)	34/66	4	0.8
0301	1202	019	15b	08	11	10	2.0
0601	1501	019	15b	08	24	6	1.2
0601	1502	019	1521	04	34/66	6	1.2
0501	1502	019	18	07 (01–03)	0203	3	0.6
0501	1502	018	18	0704	0203	5	1.0
0501	1502	018	18	0704	24	8	1.6
0301	1202	018	18	0704	24	5	1.0
0301	1101	008	27	0304	24	2	0.4
0301	1202	008	27	0304	24	3	0.6
0501	1502	008	27	0304	24	6	1.2
0501	1502	002	38	07(01–03)	11	2	0.4
0501	1502	002	38	07 (01–03)	24	2	0.4
0302	0405	008	39/6701	07 (01–03)	0203	5	1.0
02	07	004	44	07 (01–03)	33	13	2.5
0303	09	010	4601	01	11	6	1.2
0502	1202	010	4601	01	11	6	1.2
0303	09	010	4601	01	24	3	0.6
0303	09	010	4601	01	0207	21	4.1
0502	1202	010	4601	01	0207	10	2.0
0502	1401	010	4601	01	0207	4	0.8
0502	1602	010	4601	01	0207	2	0.4
0303	07	017	57	0602	01	4	0.8
02	0301	002	5801	0302	33	26	5.1
06 (04–07)	1302	002	5801	0302	33	3	0.6
02	0301	002	5801	0302	11	4	0.8

15a=15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27, 32–35)
15b=15 (02, 08, 11, 15, 28, 30)

served MICA- HLA-B – HLA-Cw haplotypes. The three most common three-locus haplotypes were MICA*010 – B*4601 – Cw*01 (HF=14.3%), MICA*02 -B*5801 – Cw*0302(HF=8.2%) and MICA*019 – B*15(02,08,11,15,28,30) – Cw*08 (HF=7.1%). The two most common six-locus haplotypes were DQB1*02 – DRB1*0301 - MICA*002 – B*5801 – Cw*0302 – A*33 (5.1%) and DQB1*0303 – DRB1*09 -MICA*010 – B*4601 – Cw*01 – A*0207 (4.1%). Further, other than those haplotypes, as many as 38 distinct three-locus and 28 six-locus haplotypes were recognized in more than two NET individuals, indicating generation of a large variety of allelic combinations among the HLA loci in NET.

Discussion

The MICA gene shows an extensive degree of genetic polymorphism, which is slightly more concentrated in the α2 domain, resembling classical HLA class I molecules. However, in contrast to classical class I molecules MICA polymorphism is equally diverse within the α3 domain. Further, sequence variation at each polymorphic position in the MICA gene has been limited to biallelic substitution in contrast to many multi-allelic ones in the classical MHC class I genes. The structure of MICA molecules revealed by X-ray crystallography confirms

the general configuration of the molecules with an MHC class I fold (20, 21). However, the MICA polymorphic residues are positioned on the outer edge of an antigen-binding cleft, apparently bordering an invariant ligand-binding site, unlike MHC class I molecules. Therefore, actual role of polymorphism in MICA molecules has yet to be determined.

In order to investigate genetic polymorphism in the extracellular domains of the MICA gene in the NET population, SBT was adopted in this study. Consequently, MICA*008 was the most common allele, but its allele frequency showed a significant decrease as compared with those of the Japanese and Caucasian populations (see Table 2). The MICA*008 allele is highly dominating in Caucasians (AF=55%), being associated with B*0801, B*0702, B*4402 and B*4001 (19). In Caucasian, its predominant abundance and representation on multiple haplotypes could indicate that MICA*008 is the ancestral allele with subsequent recombinations and point mutations producing further allelic diversity (19). In the NET population, MICA*008 (AF= 21.4%) is mostly associated with unique HLA-B alleles (B*27 and B*39/6701) different from that in the Caucasian population. MICA*008 in the NET population carries the A5.1 allele in the TM (transmembrane) domain (unpublished data), as similarly observed in other populations (16, 22), which possibly produces a truncated MICA protein because of a frame-shift mutation due to a one-base insertion in exon 5.

MICA*010 and *019, which are different from MICA*008 by double- and single-nucleotide substitutions, are also prevalent with AFs of 18.2% and 15.3% in NET, respectively. These two MICA alleles might have been generated from MICA*008 and then conserved in the Thai population. However, they were associated with TM (A5) and HLA-B alleles (B*4601 and B*15) different from MICA*008 in this population. Additionally, MICA*010 carries a nucleotide substitution at position 17 in exon 2, which results in a proline instead of an arginine by substitution at amino acid position 6 in the α1 domain. This substitution has been recently established to abolish cell surface expression of the MICA*010 allele by blocking a β-sheet hydrogen bond with the histidine carbonyl group at position 27 on the β2 strand. Moreover, this substitution is incompatible with β-sheet secondary structure, thus likely interfering with protein folding (20, 23). Thus far, we have recognized six individuals homozygous for the MICA*010 – B*4601 – Cw*01 haplotypes, and all of them were apparently healthy. This might be consistent with the fact that no MICA/MICB knock-out individuals homozygous for the MICA deletion – MICB null (MICB0107N) haplotype manifested any clinical symptoms (17, 24, 25).

Interestingly, MICA052, which is identical to MICA*007 except for a single-nucleotide substitution at nucleotide position 751 (G to C), was detected only in the NET population, but not in the Japanese or Caucasian population (see Table 2). MICA*007 was not present in NET. The MICA052 sequence was submitted to GenBank as Accession No AH007476, but its ethnic origin is unknown. This allele was in a strong linkage disequilibrium with B*13 (41/42) in NET (see Table 3). HLA-B*13, which is quite common in NET, is known to be linked to three Cw alleles as major haplotypes (B*13-Cw*0304, B*13-Cw*04 and B*13-Cw*0602). Among them, the former two-locus B*13-Cw*0304 and B*13-Cw*04 haplotypes were associated with MICA052 (see Table 4). Collectively, these facts imply that MICA052 is a relatively old allele introduced to or newly generated in the B*13-associated haplotype in NET before the diversification of the B*13-Cw haplotype. Further, the existence of a large number of the two-locus, three-locus and six-locus MICA associated haplotypes may also suggest the ancient origin of MICA alleles, possibly before recent creation of HLA diversity.

Natural killer cells have been shown to express MHC class I molecules recognizing receptors that are thought to function primarily as negative or positive signalling receptors in the course of the self-non-self discrimination. Among them, NKG2D is supposed to be the putative ligand as an activating receptor for MICA molecules. It remains to be determined whether NKG2 can distinguish MICA alleles and whether other NK receptors such as KIR2 (CD158a/b), a killer cell inhibitory receptor, can recognize two alternative epitopes on the HLA-Cw molecules (26). If so, MICA allelic distribution and/or linkage disequilibria between MICA and other class I alleles may give important information on donor-recipient matching and the outcome of transplantation. In conclusion, we carried out SBT of MICA in the NET population showing a large variety of the allelic and haplotypic distributions, which will provide the important basis for future analyses on the potential role of the MICA gene in disease susceptibility and transplantation matching in South East Asian populations.

References

1. Bahram S, Bresnahan M, Geraghty DE, Spies T. A second lineage of mammalian major histocompatibility complex class I genes. *Proc Natl Acad Sci U S A* 1994: **91**: 6259–63.
2. Leelayuwat C, Townend DC, Degli-Esposti MA et al. A new polymorphic and multicopy MHC gene family related to nonmammalian class I. *Immunogenetics* 1994: **40**: 339–51.
3. Shiina T, Tamiya G, Oka A et al. Molecular dynamics of MHC genesis unraveled by sequence analysis of the 1,796,938-bp HLA class I region. *Proc Natl Acad Sci U S A* 1999: **96**: 13282–7.

4. Gaudieri S, Leelayuwat C, Townend D et al. Allelic and interlocus comparison of the PERB11 multigene family in the MHC. *Immunogenetics* 1997: **45**: 209–16.
5. Zwirner NW, Fernandez-Vina MA, Stastny P. MICA, a new polymorphic HLA-related antigen, is expressed mainly by keratinocytes, endothelial cells and monocytes. *Immunogenetics* 1998: **47**: 139–48.
6. Leelayuwat C, Hollingsworth P, Pummer S et al. Antibody reactivity profiles following immunization with diverse peptides of the PERB11 (MIC) family. *Clin Exp Immunol* 1996: **106** (5): 68–76.
7. Groh V, Rhinehart R, Secrist H et al. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci U S A* 1999: **96**: 6879–84.
8. Groh V, Bahram S, Bauer S et al. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci U S A* 1996: **93**: 12445–50.
9. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial γδ T cells. *Science* 1998: **279**: 1737–40.
10. Mak TW, Ferrick DA. The γδ T-cell bridge: linkage innate and acquired immunity. *Nat Med* 1998: **4**: 764–5.
11. Bauer S, Groh V, Wu J et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 1999: **285**: 727–9.
12. Fodil N, Laloux L, Wanner V et al. Allelic repertoire of the human MHC class I MICA gene. *Immunogenetics* 1996: **44**: 351–7.
13. Bahram S. MIC genes: from genetics to biology. *Adv Immunol* 2000: **76**: 1–60.
14. Marsh SGE, Bodmer JG, Albert ED et al. Nomenclature for factors of the HLA system, 2000. *Tissue Antigens* 2001: **57**: 236–86.
15. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992: **39**: 225–35.
16. Katsuyama Y, Ota M, Ando H et al. Sequencing based typing for genetic polymorphisms in exons 2, 3 and 4 of the MICA gene. *Tissue Antigens* 1999: **4**: 178–84.
17. Komatsu-Wakui M, Tokunaga K, Ishikawa Y et al. MIC-A polymorphism in Japanese and a MIC-A-MIC-B null haplotype. *Immunogenetics* 1999: **49**: 620–8.
18. Komatsu-Wakui M, Tokunaga K, Ishikawa Y et al. Wide distribution of the MICA-MICB null haplotype in East Asians. *Tissue Antigens* 2001: **57**: 1–8.
19. Petersdorf EW, Shuler KB, Longton GM et al. Population study of allelic diversity in the human MHC class I-related MIC-A gene. *Immunogenetics* 1999: **49**: 605–12.
20. Li P, Willie ST, Bauer S et al. Crystal structure of the MHC class I homologue MIC-A, a γδ T cell ligand. *Immunity* 1999: **10**: 577–84.
21. Bjorkman PJ, Saper MA, Samraoui B et al. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 1987: **329**: 506–12.
22. Fodil N, Pellet P, Laloux L et al. MICA haplotypic diversity. *Immunogenetics* 1999: **49**: 557–60.
23. Li Z, Groh V, Strong RK, Spies T. A single amino acid substitution causes loss of expression of a MICA allele. *Immunogenetics* 2000: **51**: 246–8.
24. Ando H, Mizuki N, Ota M et al. Allelic variants of the human MHC class I chain-related B gene (MICB). *Immunogenetics* 1997: **46**: 499–508.
25. Ota M, Bahram S, Katsuyama Y et al. On the MICA deleted-MICB null, HLA-B*4801 haplotype. *Tissue Antigens* 2000: **56**: 268–71.
26. Frohn C, Schlenke P, Kirchner H. The repertoire of HLA-Cw-specific NK-cell receptors CD 158a/b (EB6 and GL 183) in individuals with different HLA phenotypes. *Immunology* 1997: **92**: 567–70.

Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions

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The anticonvulsant carbamazepine (CBZ) frequently causes cutaneous adverse drug reactions (cADRs), including maculopapular eruption (MPE), hypersensitivity syndrome (HSS), Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). We reported that SJS/TEN caused by CBZ is strongly associated with the *HLA-B*1502* gene in Han Chinese. Here, we extended our genetic study to different types of CBZ-cADRs (91 patients, including 60 patients with SJS/TEN, 13 patients with hypersensitivity syndrome and 18 with maculopapular exanthema versus 144 tolerant controls). We used MALDI-TOF mass spectrometry to screen the genetic association of 278 single nucleotide polymorphisms (SNPs), which cover the major histocompatibility complex (MHC) region, tumor necrosis factor-alpha, heat shock protein and CBZ-metabolic enzymes, including CYP3A4, 2B6, 2C8, 2C9, 1A2 and epoxide hydrolase 1. In addition, we genotyped 20 microsatellites in the MHC region and performed HLA-typing to construct the recombinant map. We narrowed the susceptibility locus for CBZ-SJS/TEN to within 86 kb flanking the *HLA-B* gene on the extended B*1502 haplotype, and confirmed the association of B*1502 with SJS/TEN [$P_c = 1.6 \times 10^{-41}$, odds ratio (OR) = 1357; 95% confidence interval (CI) = 193.4–8838.3]. By contrast to CBZ-SJS/TEN, *HLA-B*1502* association was not observed in the MPE or HSS groups: MPE was associated with SNPs in the HLA-E region and a nearby allele, *HLA-A*3101* ($P_c = 2.2 \times 10^{-3}$, OR = 17.5; 95% CI = 4.6–66.5), and HSS with SNPs in the motilin gene ($P_c = 0.0064$, OR = 7.11; 95% CI = 3.1–16.5) located terminal to the MHC class II genes. No SNPs in genes involved in CBZ metabolism were associated with CBZ-induced cADRs. Our data suggest that *HLA-B*1502* could contribute to the pathogenesis of CBZ-SJS/TEN, and that genetic susceptibility to CBZ-induced cADRs is phenotype-specific. *Pharmacogenetics and Genomics* 16:297–306

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Introduction

Although carbamazepine (CBZ) is a commonly prescribed first-line anticonvulsant, it is also a relatively common cause of cutaneous adverse drug reactions (cADRs) [1]. The frequency of CBZ-induced cADRs is between 1 in 1000 and 1 in 10 000 new exposures to the drug in Caucasians [2,3]. The cADRs range from mild maculopapular eruption (MPE), with increasing severity, to hypersensitivity syndrome (HSS), Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). The mortality rate of the latter can be as high as 40% [4]. MPE is characterized by cutaneous fine pink macules and

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papules, lesions which usually fade within 1–2 weeks following cessation of drug treatment. HSS is characterized by multi-organ involvement (e.g. hepatitis and nephritis) accompanied by systemic manifestations (e.g. fever, arthragias, eosinophilia and lymphadenopathy) in addition to skin rashes [4]. Skin manifestations of HSS may vary from MPE to exfoliative dermatitis [5]. HSS is also called drug rash with eosinophilia and systemic symptoms. SJS and TEN are characterized by a rapidly developing blistering exanthema of purpuric macules and target-like lesions accompanied by mucosal involvement and skin detachment [6]. According to the clinical morphology, SJS/TEN belong to the group of bullous cADRs, whereas MPE and HSS are non-bullous reactions.

The mechanism by which CBZ causes cADRs is not well understood. Potential defects in the enzymes responsible for bioactivation and detoxification of CBZ have been proposed [1]. Carbamazepine is bioactivated by hepatic cytochrome P450 enzymes, mainly CYP3A4, CYP2B6 and CYP2C8, which generate various potentially reactive metabolites, such as CBZ 10,11-epoxide, 3-hydroxy CBZ, 2-hydroxy CBZ, and CBZ 2,3-epoxide [7,8]. Most of the reactive epoxides are detoxified to non-toxic dihydrodiols by liver microsomal epoxide hydrolase 1 (EPHX1) or to glutathione conjugates by glutathione transferase [9,10]. Previous attempts to identify a genetic defect altering the structure or function of epoxide hydrolase 1 in individuals susceptible to CBZ-induced cADRs, however, were not successful [11,12].

It has also been proposed that immune reactions are involved in the cADRs triggered by CBZ because infiltrating inflammatory cells can be detected in the skin lesions, and rechallenging with the same drug typically shortens the incubation period, resulting in more severe manifestations [13,14]. CD4 + T cells are the major cell type found in the skin lesions of MPE and HSS [15], whereas CD8 + T-cell-mediated cytotoxic responses appear to be the major event in SJS/TEN [16]. There is also evidence supporting the view that cADRs involve major histocompatibility complex (MHC)-dependent presentation of its metabolites for T cell activation [16,17]. Naisbitt *et al.* [15] reported that CBZ might be presented by MHC class II expressed on the surface of antigen-presenting cells to the T-cell receptor of CD4 + T cells of patients with CBZ-induced HSS [15].

We recently reported that, in Han Chinese, the *HLA-B*1502* gene is strongly associated with CBZ-induced SJS and TEN [18]. In the present study, we sought to extend our genetic association study to other types of cADRs induced by CBZ using a comprehensive genetic screen of markers in the MHC region, as well as genes encoding CBZ metabolic enzymes. In addition, we performed fine mapping to further investigate the susceptibility locus for CBZ-induced SJS/TEN.

Materials and methods

Study population

From 1997 to 2004, 91 individuals who fulfilled diagnostic criteria for CBZ-induced MPE, HSS, SJS or TEN agreed to participate in the study. Eighty-eight of the 91 patients were recruited from Chang Gung Memorial Hospital Health System, Veterans General Hospital, National Taiwan University Hospital, Cathay General Hospital and Chushang Show-Chwan Hospital, Taiwan. We recruited patients primarily from inpatients who had more severe diseases. The remaining three patients were referred by physicians from Hong Kong (two patients) and the USA (one patient). The latter three all had CBZ-SJS. All patients (including the three from Hong Kong and the USA) and controls (see below) were Han Chinese or Chinese descendants.

The 91 patients included 44 patients with CBZ-SJS/TEN who we previously reported [18], and 16 additional patients with SJS/TEN, as well as 13 patients with hypersensitivity syndrome and 18 with maculopapular exanthema. All patients were assessed by two dermatologists who reviewed photographs, pathological slides and medical records. Diagnostic criteria for SJS/TEN were based on the clinical morphology defined by Roujeau [6]. We defined 'SJS' as a skin detachment of less than 10% of total body-surface area, 'overlap of SJS and TEN' as skin detachment of 10–30%, and 'TEN' as skin detachment greater than 30%. The criteria for HSS in this study were skin rash, plus two of the following symptoms: fever, lymphadenopathy and haematologic abnormalities (e.g. eosinophilia, atypical lymphocytosis) with involvement of at least one internal organ (e.g. hepatitis, pneumonitis, myocarditis, pericarditis, nephritis) [2,5,19]. SJS and TEN are bullous cADRs and are considered to be variants of the same disease. Therefore, they were analysed together as a group. MPE and HSS are non-bullous and it is less clear whether they are from a single disease spectrum. They were therefore analysed as a group and also analysed separately as two entities. In all enrolled cases, CBZ was regarded as the offending drug if the onset of cADRs symptoms occurred within the first 2 months of exposure and the symptoms resolved upon withdrawal of the drug. Patients with an absence of symptoms after re-exposure to CBZ were excluded.

The control group was the 144 consecutive patients who received CBZ for at least 3 months without evidence of adverse reactions. These tolerant patients were recruited from the Neurology Clinic of the same regional hospitals where cADRs patients were recruited. In addition, 93 healthy subjects were randomly selected from a biobank under a nationwide population study, in which 3312 Han

Chinese descendants were recruited based on the geographical distribution across Taiwan. There was no self-report of adverse events in any of these 93 subjects. The study was approved by the institutional review board, and informed consent was obtained from all of the participants.

DNA isolation and genotyping

Genomic DNA was isolated using the PUREGENE DNA purification system (Gentra Systems, Minneapolis, Minnesota, USA).

Short tandem repeat polymorphism (STRP) genotyping

Twenty highly polymorphic microsatellite markers located in the MHC region were selected from the NCBI database (i.e. D6S258, D6S2972, D6S510, D6S265, D6S388, D6S2814, HLAC-CA, HLABC-CA, MIB, MICA, TNFd, BAT2-CA, D6S273, D6S1615, DQCAR, G51152, D6S2414, D6S1867, D6S1560 and D6S1583). The average heterozygosity of markers was 0.702 with an estimated 230 kb of spacing. Primers were designed based on oligonucleotide sequences reported within the database. Polymerase chain reaction (PCR) for genotyping was performed in a 5-μl volume containing 10 ng of genomic DNA and 0.33 μM of each primer by using GeneAmp 9700 thermocyclers (Applied Biosystems, Foster City, California, USA). Up to six products of appropriate size and carrying a fluorescent label were pooled for capillary gel electrophoresis. The size of polymorphic amplicons was determined by ABI 3730 DNA sequencer (Applied Biosystems) using the LIZ500 size standard as an internal size standard. Allele sizing was calculated using the GENMAPPER program version 3.0 (Applied Biosystems). Allele calling and binning were performed using the SAS program (SAS Institute, Cary, North Carolina, USA). Three CEPH control individuals (1331-01, 1331-02, 1347-2) and H_2O were included in all genotyping experiments for quality control purposes.

Single nucleotide polymorphisms (SNP) genotyping on MHC region and metabolic enzymes for CBZ

A total of 379 SNPs were selected from the SNP database (dbSNP: build 123) of the National Center for Biotechnology Information for genotyping [20]. These included 220 SNPs from 4 Mb of the MHC region on chromosome 6p21.3 and 159 SNPs selected from genes encoding drug metabolizing enzymes. The 220 SNPs selected in the MHC region included 201 SNPs reported by Walsh *et al.* [21], and additionally included SNPs of tumor necrosis factor (TNF)-alpha and the heat shock proteins. We especially included rs1800629, TNF-308A allele, which is reported to be associated with CBZ-HSS [22], and rs2227956 of HspA1L (heat shock 70 kDa protein1-like), which is reported to be associated with abacavir hypersensitivity [23]. The average space of selected SNPs in the MHC region was approximately 20 kb. Another 159 SNP set contained genes potentially involved in bioactivation or detoxification of CBZ metabolism, including CYP3A4, CYP2B6, CYP2C8, CYP2C9, CYP1A2 and EPHX1.

SNP genotyping was performed using high-throughput MALDI-TOF mass spectrometry. Briefly, primers and probes were designed using SpectroDESIGNER software (Sequenom, San Diego, California, USA). Multiplex PCRs were performed, and unincorporated dNTPs were dephosphorylated using shrimp alkaline phosphatase (Hoffman-LaRoche, Basel, Switzerland) followed by primer extension. The purified primer extension reaction was spotted onto a 384-element silicon chip (SpectroCHIP, Sequenom), analysed in the Bruker Biflex III MALDI-TOF SpectroREADER mass spectrometer (Sequenom) and the resulting spectra processed with SpectroTYPER (Sequenom).

Of the 379 SNPs selected from the database, 101 SNPs were excluded from further analysis due to the non-polymorphism, low successful rate (less than 0.7), and/or departure from Hardy–Weinberg equilibrium ($P < 0.001$) in our control group. The remaining 278 SNPs, including 88 SNPs in metabolic pathway for CBZ and 190 SNPs in the MHC region, were used for the statistical analyses and the *P*-values of association were adjusted by using Bonferroni correction.

HLA genotyping

HLA alleles *A*, *B*, *C* and *DRB1* were determined by sequence-specific oligonucleotide reverse lineblots (DYNAL Biotech Ltd, Bromborough, UK) [24]. Potential ambiguities were resolved by sequencing-based typing [25]. The primers used for PCR amplification of DNA fragments of *HLA-A*, *B* and *C* genes were: (i) Bin1-TA-M13F, Bin1-CG-M13F and Bin3-M13R for *HLA-B* alleles; (ii) Ain1-A-M13F, Ain1-G-M13F, Ain1-T-M13F and Ain3–62-M13R for *HLA-A* alleles; and (iii) 5Cln1-61 and 3BCln3-12 for *HLA-C* alleles [26]. The *DRB1* alleles were separated into eight groups: DR1, DR2, DR3/11/6, DR4, DR7, DR8/12, DR9, DR10 for sequencing-based typing as described [27]. Genotyping for MHC class I chain-related gene A (MICA) was carried out by DNA sequencing on its exons 2, 3, 4 and 5 [25]. The primers used for PCR amplification of the DNA fragment of the *MICA* gene were: forward primer: 5′-CGTTCTTGTCCCTTTGCCCGTGTGC-3′ and reverse primer: 5′-GATGCTGCCCCATTCCCTTCCCAA-3′. The sequence data were analysed by SeqScape v2 (Applied Biosystems).

Statistical analysis

Comparisons of allele or genotype frequencies between groups were performed using Fisher's exact tests. All *P*-values were two-tailed. $P < 0.05$ was considered to be statistically significant. An allelic association screen was

carried out by the Cochran–Armitage Trend test for each SNP or STRP marker [28]. To test the association of haplotype frequencies, the composite haplotype method and haplotype trend regression of Helix Tree software version 3.0.0 were used. The linkage disequilibrium between two loci was analysed by using Expectation/Maximization (EM) method provided by Helix Tree software version 3.0.0 (Golden Helix Inc., Bozeman, Minnesota, USA). To achieve sufficient power to identify loci associated with different clinical manifestations, the corrected P (P_c) values were adjusted by using Bonferroni's correction for multiple comparisons (278 for SNP assays, 17 for HLA-A, 40 for HLA-B, 19 for HLA-C, 30 for HLA-DRB1, 25 for MICA, and 20 for HLABC-CA). Odds ratios (ORs) were calculated using Haldane's modification, which adds 0.5 to all cells to accommodate possible zero counts [29].

Results

Characteristics of patients and controls

Based on the clinicopathologic features of cADRs, our patients could be divided into bullous and non-bullous groups: (i) bullous: CBZ-SJS/TEN ($n = 60$; 54 with SJS, five with overlapping SJS/TEN, one with TEN) and (ii) non-bullous: CBZ-MPE/HSS ($n = 31$; 18 with MPE, 13 with HSS). The non-bullous group was further separated into two subgroups (MPE and HSS) for data analysis. Clinical manifestations and demographic variables of the 91 patients and 144 tolerant controls are summarized in Table 1. The onset of symptoms for all patients with cADRs occurred within the first 2 months of CBZ exposure. The mean duration of CBZ exposure was longer in HSS patients than in SJS/TEN. Three patients with SJS/TEN had a second attack within 2 days of re-exposure; one of them developed TEN and died during the second attack. All 60 patients with SJS/TEN had widespread purpuric rash with blisters and mucosal involvement. In addition to maculopapular exanthema, all 13 patients with HSS had symptoms of multi-organ or systemic involvement.

One hundred and forty-four patients who had been on CBZ for at least 3 months (mean 75.03 months) with no self-report of adverse events were used as tolerant controls. Their demographic variables are shown in Table 1. Note that the CBZ-tolerant controls received higher doses of CBZ (mean dosage 793.8 mg/day) and yet no adverse drug reactions were observed.

Fine mapping of the major histocompatibility complex region for genetic susceptibility to CBZ-SJS/TEN

To confirm our previous observation of the genetic association between *HLA-B*1502* and CBZ-SJS/TEN, and to further define the susceptibility region, we genotyped 20 STRP markers and 190 SNPs in the MHC region. Nine STRP markers located between D6S2814 and D6S2414 (physical map 30.8–32.9 Mb, chromosome 6) showed significant association (P-values $< 10^{-6}$; Fig. 1). In particular, HLABC-CA near *HLA-B* showed the strongest association ($P = 3.4 \times 10^{-19}$; Fig. 1), followed by D6S1615, a marker closed to *HLA-DRB1* locus.

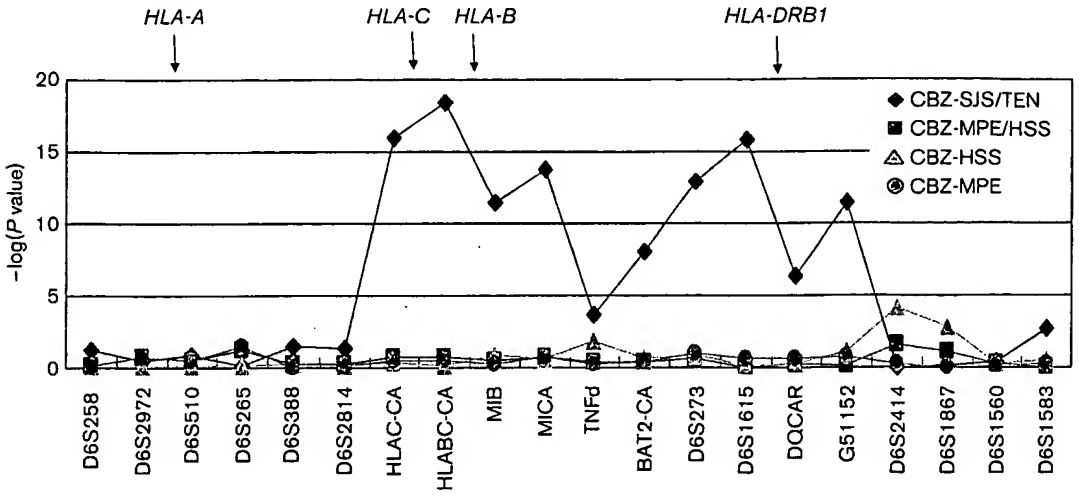
Thirty-seven SNPs showed P-values of less than 0.001 (Fig. 2a). Among them, seven SNPs located between HLA-DRA and HLA-C (physical position 31.3–32.5 Mb, chromosome 6) showed strong associations ($P < 10^{-10}$) (Fig. 2a). The three most important SNPs were rs3130690, rs2848716 and rs750332, located near *HLA-B*, *MICA* and *BAT3* genes (Table 2). The rs3130690, an intergenic SNP with 36 kb telomeric to the *HLA-B* locus, demonstrated the strongest association with CBZ-induced SJS/TEN ($P_c = 1.29 \times 10^{-39}$) (Table 2). The T allele of the rs3130690 SNP was present in 95% (57/60) of CBZ-SJS/TEN patients, but only in 6.9% (10/144) of

Table 1 Demographic variables, dosage and duration of carbamazepine (CBZ) exposure, and clinical characteristics in CBZ-induced cutaneous adverse reactions and CBZ-tolerant controls

	Bullous cADRs	Non-bullous cADRs ($n=31$)		
	SJS/TEN ($n=60$)	HSS ($n=13$)	MPE ($n=18$)	Tolerant controls ($n=144$)
Sex, *n* (%)				
Male	33 (55)	9 (69.2)	8 (44.4)	75 (52.1)
Female	27 (45)	4 (30.8)	10 (55.6)	69 (47.9)
Age (years), mean (range)	43.4 (5–80)	51.5 (6–83)	45.9 (7–84)	35.7 (5–79)
CBZ exposure, mean (range)				
Dosage (mg/day)	332.6 (100–600)	420 (300–600)	423.1 (200–800)	793.8 (100–1500)
Duration	15.1 (2–49) days	32.7 (15–54) days	22.4 (7–55) days	75.03 (3–287.5) months
Cutaneous features, *n* (%)				
Blister or epidermal detachment	60 (100)	0 (0)	0 (0)	Not observed
Mucosal erosions	60 (100)	3 (23)	0 (0)	Not observed
General and laboratory, *n* (%)				
High fever (>38.5°C)	41 (68.3)	12 (92.3)	11 (61.1)	Not observed
Eosinophil count >1000/mm^3	4 (6.7)	10 (76.9)	4 (22.2)	Not observed
Atypical lymphocytosis	8 (13.3)	7 (53.8)	3 (16.7)	Not observed
Abnormal liver function	7 (11.7)	11 (84.6)	0 (0)	Not observed
Abnormal renal function	1 (1.7)	2 (15.4)	0 (0)	Not observed

SJS/TEN, Stevens–Johnson syndrome/toxic epidermal necrolysis; HSS, hypersensitivity syndrome; MPE, maculopapular exanthema.

Fig. 1



Association screen of 20 short tandem repeat polymorphism (STRP) markers in the major histocompatibility complex (MHC) region with carbamazepine (CBZ)-induced cutaneous adverse drug reactions. On the *x*-axis, 20 STRP markers in the MHC region are ordered by their physical positions (29.9–33.9 Mb) on chromosome 6p21.3. On the *y*-axis, the $-\log_{10} P$ values were calculated by comparison of the allele frequencies between the patients and tolerant group using the Cochran–Armitage exact trend test. Genotyping data of four groups of patients are presented: diamond symbols: CBZ-induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN); square symbols: CBZ-induced maculopapular eruption (MPE)/hypersensitivity syndrome (HSS); triangle symbols: CBZ-induced HSS; and circle symbols: CBZ-induced MPE.

tolerant controls [OR = 254.6; 95% confidence interval (CI) = 70.4–901.9] for TT/TG genotype versus GG genotype) (Fig. 3). To verify the results of our association screen, we also genotyped 93 healthy subjects randomly selected from the general population [30]. A strong association between SNPs and CBZ-induced SJS/TEN was replicated in the MHC region, especially rs3130690, nearby the *HLA-B* locus (data not shown).

We further genotyped the individual *HLA-A*, *B*, *C*, *DRB1* and *MICA* alleles. The *HLA-B*1502* allele was present in 98.3% (59/60) of CBZ-SJS/TEN patients, whereas only 4.2% (6/144) of the tolerant controls were positive for the allele ($P_c = 1.6 \times 10^{-41}$, OR = 1357; 95% CI = 193.4–8838.3) (Table 3 and Fig. 3). The only CBZ-SJS patient who did not have the *B*1502* allele, had instead, another HLA-B15 allele: *HLA-B*1558*. In addition to the positive association, we also found that *HLA-B*4001* was negatively associated with CBZ-SJS/TEN ($P_c = 2.6 \times 10^{-4}$, OR = 0.16; 95% CI = 0.1–0.4) (Table 3). The *HLA-Cw*0801* and *MICA*019* alleles flanking the *HLA-B*1502* showed strong linkage disequilibrium with *HLA-B*1502*, and were present in 93.3% (56/60) and 95% (57/60) of SJS/TEN patients, respectively. An extended B*1502 haplotype formed by polymorphic alleles (A*1101-Cw*0801-HLABC-CA*119-rs3130690T-B*1502-MICA*019-DRB1*1202) had a strong association with CBZ-induced SJS/TEN. The recombinant map of Cw*0801-HLABC-CA*119-rs3130690T-B*1502-MICA*019 defined the susceptible region within 86 kb (i.e. between the T allele of rs3130690 and *MICA*019*) flanking the *B*1502* gene in the 4 Mb MHC region (Fig. 3). Within this 86-kb region, *HLA-B* is the only known gene. Taken together, the data suggested that one or more alleles in the vicinity of the *HLA-B* locus, particularly *B*1502* itself, participate in the pathogenesis of CBZ-induced SJS/TEN.

Association screen for candidate gene SNPs with CBZ-MPE/HSS

When MPE and HSS were grouped together as non-bullous cADRs, no STRP markers showed association, and only six SNPs had *P*-values less than 0.01 (Figs 1 and 2b). The three most significant SNPs are listed in Table 2, including rs1264511 located near HLA-E, rs1042389 located on the 3′-untranslation region of CYP2B6, and rs2894342 located in the promoter region of the motilin (*MLN*) gene (Table 2). However, the *P*-values became non-significant after correcting for multiple testing (278 SNP assays) was performed.

In HLA-typing, *HLA-A*3101* showed an association with MPE/HSS ($P_c = 0.0021$) (Table 3). *HLA-A*3101* was present in 25.8% (8/31) of patients with MPE/HSS, but only in 2.8% (4/144) of tolerant controls (OR = 12.17; 95% CI = 3.6–41.2).

Association between *MLN* polymorphisms and HSS

When genetic analysis was performed separately for HSS and MPE, one STRP marker, D6S2414 (physical map: 32.9 Mb, chromosome 6) showed an association with

Table 2 Association between the three most significant single nucleotide polymorphism (SNP) alleles and carbamazepine (CBZ)-induced cutaneous adverse reactions

Groups	Reference SNP	Chromosomal position	Gene symbol	Location	Allele type	Allele frequency		P-value	P_c value	Odds ratio (95% confidence interval)
						Case	Controls [a]			
SJS/TEN ($n=60$)	rs3130690	6:31393914	Near HLA-B	Intergene	T/G	T 0.683	0.045	4.66×10^{-42}	1.29×10^{-39}	45.65 (23.34–89.13)
	rs2848716	6:31495946	MICA	3′-UTR	C/G	C 0.691	0.246	7.58×10^{-17}	2.10×10^{-14}	6.86 (4.29–10.96)
	rs750332	6:31715029	BAT3	Intron	C/T	C 0.508	0.139	3.04×10^{-14}	8.45×10^{-12}	6.41 (3.94–10.44)
MPE/HSS ($n=31$)	rs1264511	6:30521951	Near HLA-E	Intergene	G/C	G 0.274	0.108	0.001685	NS	3.13 (1.61–6.09)
	rs1042389	19:46215993	CYP2B6	3′-UTR	C/T	C 0.516	0.299	0.001696	NS	2.51 (1.44–4.36)
	rs2894342	6:33882372	MLN	Promoter	A/C	A 0.258	0.108	0.00342	NS	2.88 (1.47–3.66)
HSS ($n=13$)	rs2894342	6:33882372	MLN	Promoter	A/C	A 0.462	0.108	0.0000232	0.0064	7.11 (3.07–16.51)
	rs2075800	6:31885925	HSPA1L	Coding	G/A	G 0.923	0.622	0.001173	NS	7.31 (1.87–28.37)
	rs2395402	6:33861651	LEMD2	Intron	G/A	G 0.423	0.160	0.002381	NS	3.86 (1.69–8.80)
MPE ($n=18$)	rs1264511	6:30521951	Near HLA-E	Intergene	G/C	G 0.333	0.108	0.0007862	NS	4.15 (1.91–9.02)
	rs1059510	6:30565711	HLA-E	Coding	T/C	T 0.472	0.243	0.005176	NS	2.79 (1.39–5.60)
	rs986475	6:31664688	LST1	5′-UTR	C/T	C 0.278	0.108	0.007597	NS	3.19 (1.43–7.15)

[a]144 tolerant-controls were genotyped. The association of allele frequencies was examined by Fisher's exact test and the P_c values were adjusted by using Bonferroni's correction for multiple comparisons of 278 SNP assays. NS, not significant ($P>0.05$). SJS/TEN, Stevens–Johnson syndrome/toxic epidermal necrolysis; MPE, maculopapular exanthema; HSS, hypersensitivity syndrome.

CBZ-induced HSS ($P = 6.25 \times 10^{-5}$, Fig. 1). In addition, several SNPs located in the MHC class II terminal region, around the motilin gene (physical map: 33.8 Mb), showed associations with HSS (Table 2 and Fig. 2c). The most significant SNP rs2894342 was located in the promoter of the *MLN* gene, and its A allele had an increased risk for CBZ-HSS ($P_c = 0.0064$, OR = 7.11; 95% CI = 3.1–16.5) (Table 2). The next most significant SNP was a nonsynonymous SNP (rs2075800 of HSPA1L, heat shock 70 kDa protein1-like) involving an A to G transition which leads to an amino acid change of lysine to glutamate at residue 602. The third most significant SNP was rs2395402, which was located in the intron of the LEM domain containing 2 (LEMD2) gene, 21 kb telomeric to rs2894342 of the *MLN* gene (Table 2). However, the *P*-values for the latter two SNPs became non-significant after Bonferroni's correction.

None of the *HLA-A*, *B*, *C* and *DRB1* alleles had significant associations with CBZ-induced HSS (Table 3).

Association between *HLA-A*3101* and MPE

STRP or SNP markers in the *HLA-B* or *MLN* region on chromosome 6p21.3 showed no association with MPE (Figs 1 and 2d). Instead, two SNPs near the HLA-E region (physical position 30.5 Mb) and one SNP in the 5′-untranslation region of the leukocyte specific transcript 1 (LST1) gene showed associations with MPE (Fig. 2d and Table 2, $P = 0.00078$–0.0075). The two SNPs in the HLA-E region were: rs1264511, an intergenic SNP located near HLA-E and rs1059510 located in HLA-E gene (Table 2). HLA-genotyping further revealed that the *HLA-A*3101* allele, approximately 530 kb telomeric to HLA-E, was present in 33.3% (6/18) of MPE patients but only in 2.8% (4/144) in the tolerant control group ($P_c = 2.2 \times 10^{-3}$, OR = 17.5; 95% CI = 4.6–66.5) (Table 3).

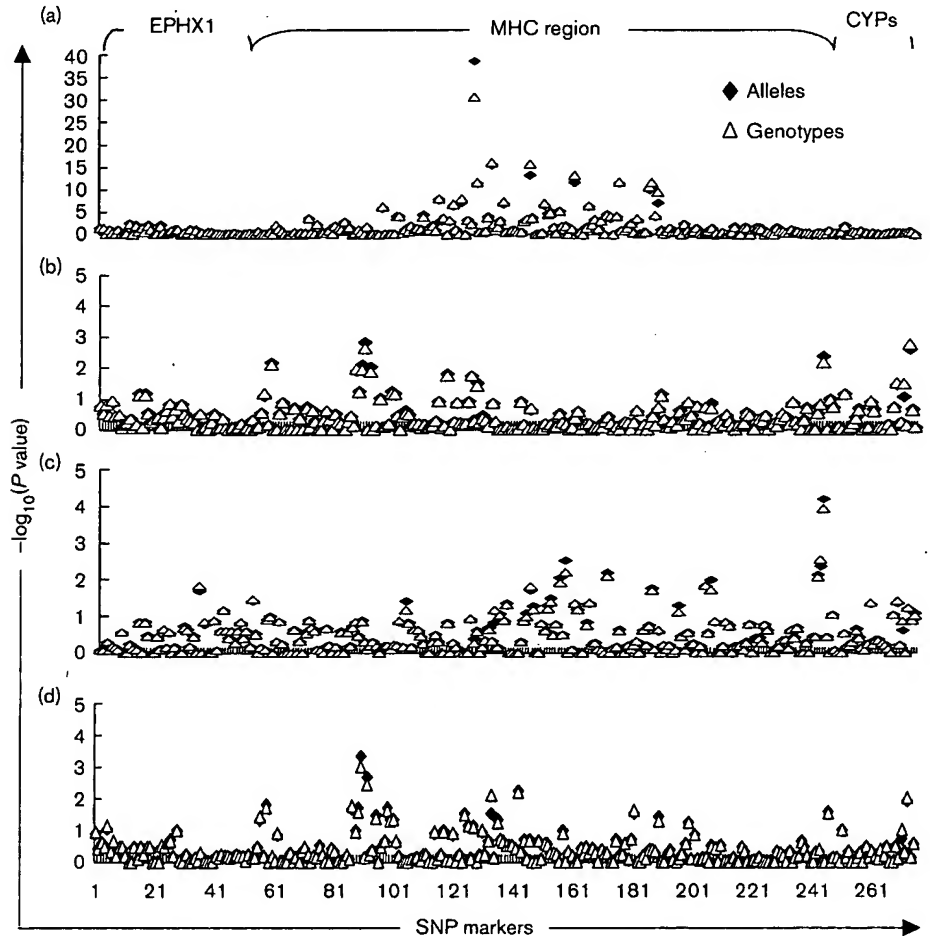
Association screen for SNPs in drug metabolizing enzymes

Genotyping of 88 informative SNPs in genes that are potentially involved in metabolic pathways for CBZ, including CYP3A4, CYP1A2, CYP2C8, CYP2B6, CYP2C9 and EPHX1, in various types of CBZ-induced cADRs, showed that only one SNP, rs1042389, in the 3′-untranslation region of CYP2B6, had a weak association with CBZ-MPE/HSS ($P = 0.0016$, Table 2 and Fig. 2b). The *P*-value became non-significant after Bonferroni's correction, suggesting that the genetic polymorphisms of genes encoding drug metabolizing enzymes have no significant association with CBZ-induced cADRs, regardless of the type of cADRs.

Discussion

This study confirmed our previous observation that *HLA-B*1502* is strongly associated with CBZ-SJS/TEN in Han

Fig. 2



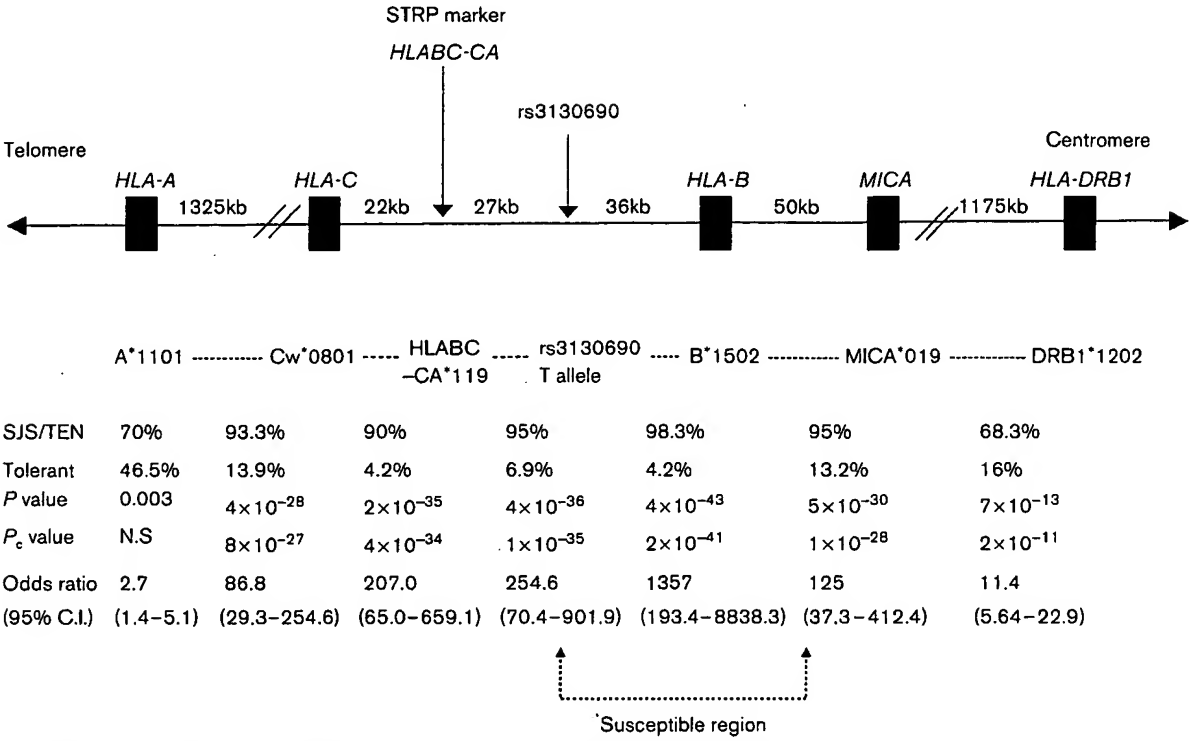
Screening of 278 candidate single nucleotide polymorphisms (SNPs) for association with (a) carbamazepine (CBZ)-induced Stevens–Johnson syndrome/toxic epidermal necrolysis, (b) CBZ-induced maculopapular eruption (MPE)/hypersensitivity syndrome (HSS), (c) CBZ-induced HSS and (d) CBZ-induced MPE. On the *x*-axis, 278 SNPs are ordered by their chromosomal positions, including 58 SNPs of epoxide hydrolase 1 (EPHX1) on chromosome 1, 190 SNPs in the major histocompatibility complex (MHC) region on chromosome 6, and 30 SNPs of CYP3A4, 2C8, 2C9, 1A2 and 2B6. On the *y*-axis, the $-\log_{10}$ *P*-values were calculated by comparison of the allele (diamond symbols) or genotype (triangle symbols) frequencies between the patients and tolerant group using the Cochran–Armitage exact trend test.

Chinese [18]. We increased the number of study patients from the original 44 to 60 and included patients from a wide geographical distribution (Taiwan, Hong Kong, China and the USA). However, all patients enrolled were Chinese or Chinese descendants. The fact that the *HLA-B*1502* allele was present in low frequency in Caucasians may explain the low incidence of CBZ-SJS/TEN in Caucasians [31,32].

It is interesting to note that the only CBZ-SJS/TEN patient who did not have the *HLA-B*1502* gene had *HLA-B*1558*, another HLA-B15 variant. This variant allele was present in low frequency in our population (approximately 0.9%) and was not detected in any of the 144 tolerant patients. The detection of *B*1558* in one of our patients may imply that *B*1558* shares a similar structural feature with *B*1502* for triggering the immune reaction of SJS caused by CBZ.

Fine recombinant genetic mapping on the extended *HLA-B*1502* haplotype (A*1101-Cw*0801-HLABC-CA*119-rs 3130690T-B*1502-MICA*019-DRB1*1202) further narrowed

Fig. 3



Susceptible region for carbamazepine (CBZ)-induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) on the extended *HLA-B*1502* haplotype. Schematic map of 4 Mb major histocompatibility complex region on chromosome 6p21.3 shows the relative positions of *HLA-A, B, C, DRB1* and *MICA* genes, STRP marker (HLABC-CA), and SNP marker (rs3130690). The percentage of presence of the specific allele in CBZ-SJS/TEN patients and tolerant-controls is shown. The susceptible region should be within 86 kb (i.e. between T allele of rs3130690 and *MICA*019*). Within this region, *HLA-B*1502* is the only known gene and shows the strongest association with CBZ-induced SJS/TEN. NS, Not significant ($P > 0.05$).

Table 3 Association of HLA alleles with carbamazepine (CBZ)-induced cutaneous adverse reactions

	Tolerant controls ($n=144$)	SJS/TEN ($n=60$)	MPE/HSS ($n=31$)	HSS ($n=13$)	MPE ($n=18$)
HLA-A					
**1101*	67	42 [NS; 2.68 (1.4–5.1)]	12 [NS; 0.73 (0.3–1.6)]	6 [NS; 0.99 (0.3–3)]	6 [NS; 0.58 (0.2–1.6)]
**2402*	41	5 [0.026; 0.23 (0.1–0.6)]	9 [NS; 1.03 (0.5–2.4)]	3 [NS; 0.75 (0.2–2.7)]	6 [NS; 1.26 (0.5–3.5)]
**3101*	4	1 [NS; 0.59 (0.1–4.1)]	8 [0.0021; 12.17 (3.6–41.2)]	2 [NS; 6.36 (1.2–33.9)]	6 [2.2×10^{-3}; 17.5 (4.6–66.5)]
HLA-B					
**1502*	6	59 [1.6×10^{-41}; 1357 (193.4–8838.3)]	1 [NS; 0.77 (0.1–5.1)]	0 [NS; 0.79 (0.1–8.8)]	1 [NS; 1.35 (0.2–9.3)]
**4001*	59	6 [2.6×10^{-4}; 0.16 (0.1–0.4)]	10 [NS; 0.69 (0.3–1.5)]	3 [NS; 0.43 (0.1–1.5)]	7 [NS; 0.92 (0.4–2.4)]
HLA-Cw					
**0102*	54	8 [8.6×10^{-3}; 0.26 (0.1–0.6)]	14 [NS; 1.4 (0.6–3.0)]	5 [NS; 1.04 (0.3–3.2)]	9 [NS; 1.67 (0.6–4.3)]
**0801*	20	56 [7.8×10^{-27}; 86.8 (29.3–254.6)]	2 [NS; 0.43 (0.1–1.8)]	0 [NS; 0.23 (0–2.3)]	2 [NS; 0.78 (0.2–3.3)]
HLA-DRB1					
**0405*	25	1 [0.03; 0.08 (0.01–0.5)]	8 [NS; 1.66 (0.7–4.1)]	1 [NS; 0.40 (0–2.5)]	7 [NS; 3.03 (1.1–8.4)]
**1202*	23	41 [2.3×10^{-11}; 11.4 (5.6–22.9)]	5 [NS; 1.01 (0.4–2.8)]	3 [NS; 1.58 (0.4–5.8)]	2 [NS; 0.66 (0.2–2.8)]

Data are genotype, *n* of positive subjects [P_c value; odds ratio (95% confidence interval)]. The association of HLA-alleles was examined by Fisher's exact test and the P_c values were adjusted by using Bonferroni's correction for multiple comparisons (17 for HLA-A, 40 for HLA-B, 19 for HLA-C and 30 for HLA-DRB1). NS, Not significant ($P > 0.05$). SJS/TEN, Stevens–Johnson syndrome/toxic epidermal necrolysis; MPE, maculopapular exanthema; HSS, hypersensitivity syndrome.

down the susceptibility region for CBZ-SJS/TEN to within 86 kb flanking the *HLA-B* gene. In this region, *HLA-B* is the only gene present. Thus, it is most likely that *B*1502* itself is the susceptibility gene for CBZ-SJS. Furthermore, it has been proposed that the molecular mechanism causing SJS/TEN involves CD8+ cytotoxic T-cell-mediated cell death [16]. The *HLA-B* gene can elicit immune responses by presenting endogenous antigens to the cytotoxic T cells and it has been proposed that the drug and/or its metabolites may bind to the peptides which are then presented by the *HLA-B* allele and recognized by the specific T cells [33,34]. Our

cytotoxic assays also supported the view that *HLA-B*1502* is functionally involved in the cytotoxic response mediated by the activated T cells in CBZ-induced SJS (unpublished data). The recent findings of *HLA-B*5701* in abacavir hypersensitivity [35] and *HLA-B*5801* in allopurinol-induced severe cADRs [26] further support the important role of the *HLA-B* gene in these serious drug-induced adverse conditions.

SJS and TEN are cADRs characterized by bullous lesions. We observed a single patient who developed a bullous fixed drug eruption while on CBZ. Interestingly, this patient was also positive for *HLA-B*1502* (data not shown). However, the present study clearly showed that non-bullous cADRs caused by CBZ are not linked to the *HLA-B*1502* marker, suggesting that the genetic association of CBZ-cADRs is phenotype-specific. It should be pointed out that our previous study of allopurinol induced cADRs showed that both SJS/TEN and HSS were associated with a same *HLA-B* allele [26]. However, in this study, CBZ-HSS is not linked to the *HLA-B* marker as SJS/TEN. At the present time, we do not know why this discrepancy occurs. Other genetic or environmental factors may contribute and lead to various spectrum of adverse reactions caused by different drugs.

Although there is no direct evidence that MPE and HSS are the same disease spectrum with differences in severity, HSS and MPE may be grouped together as non-bullous cADRs because of the similarity of the cutaneous manifestation [2,5,19]. However, the present study suggests that HSS and MPE are two disease entities because they are linked to different genetic markers: CBZ-MPE was associated with *HLA-A*3101*, and CBZ-HSS with the motilin gene polymorphisms in the MHC class II terminal region (Figs 1 and 2 and Tables 2 and 3). We did not observe the association of CBZ-HSS with the TNF2 allele (rs1800629; TNF-308A allele; physical position: 31.6 Mb; Fig. 2c) or the ancestral haplotype 8.1 on the MHC, as reported previously in a study of CBZ-induced HSS in Caucasians [22]. This could be due to different study populations or different criteria in delineating the clinical phenotypes. In the present study, the SJS/TEN patients outnumber the patients with MPE as a reflection of the recruitment process because we recruited patients primarily from inpatients who had more severe diseases. However, in Han Chinese, patients with CBZ-SJS/TEN were indeed more frequently seen than CBZ-HSS (data not shown). Because of the small numbers of the patients enrolled in this study, further studies with a larger sample size will be needed to confirm this initial observation.

Naisbitt *et al.* [15] cloned T cells of patients with CBZ-MPE/HSS and observed that T cells may recognize CBZ depending on the presence of HLA class II (DR/DQ)-matched antigen-presenting cells. Interestingly, in our SNP and STRP screen and HLA typing data, we observed associations between CBZ-induced HSS and polymorphisms markers located terminal to MHC class II region. However, the association of *HLA-A*3101* with MPE appears in disagreement with the observation that CD4+ T cells are the major cell type found in the skin lesions of MPE [15]. This could suggest that the gene associated with MPE lies in the vicinity of the *HLA-A* locus which is in linkage disequilibrium with *HLA-A*3101*.

In conclusion, our data show that genetic susceptibility to CBZ-induced cADRs is phenotype-specific. The susceptibility gene for CBZ-induced SJS/TEN lies within an 86 kb region flanking the HLA-B locus and *HLA-B*1502* itself could be directly involved in the pathogenesis of CBZ-induced SJS/TEN. The tight association of *HLA-B*1502* and CBZ-SJS/TEN provides a plausible basis for the development of such a test to identify individuals at risk for this potentially life-threatening condition caused by CBZ in Han Chinese, as well as for a further increased understanding of the pathogenesis of the clinical syndrome.

References

1 Leeder JS. Mechanisms of idiosyncratic hypersensitivity reactions to antiepileptic drugs. *Epilepsia* 1998; **39 (Suppl 7)**:S8–S16.
2 Vittorio CC, Muglia JJ. Anticonvulsant hypersensitivity syndrome. *Arch Intern Med* 1995; **155**:2285–2290.
3 Tennis P, Stern RS. Risk of serious cutaneous disorders after initiation of use of phenytoin, carbamazepine, or sodium valproate: a record linkage study. *Neurology* 1997; **49**:542–546.
4 Roujeau JC, Stern RS. Severe adverse cutaneous reactions to drugs. *N Engl J Med* 1994; **331**:1272–1285.
5 Roujeau JC. Clinical heterogeneity of drug hypersensitivity. *Toxicology* 2005; **209**:123–129.
6 Roujeau JC. The spectrum of Stevens–Johnson syndrome and toxic epidermal necrolysis: a clinical classification. *J Invest Dermatol* 1994; **102**:28S–30S.
7 Kerr BM, Thummel KE, Wurden CJ, Klein SM, Kroetz DL, Gonzalez FJ, *et al.* Human liver carbamazepine metabolism. Role of CYP3A4 and CYP2C8 in 10,11-epoxide formation. *Biochem Pharmacol* 1994; **47**:1969–1979.
8 Madden S, Maggs JL, Park BK. Bioactivation of carbamazepine in the rat *in vivo*. Evidence for the formation of reactive arene oxide(s). *Drug Metab Dispos* 1996; **24**:469–479.
9 Bellucci G, Berti G, Chiappe C, Lippi A, Marioni F. The metabolism of carbamazepine in humans: steric course of the enzymatic hydrolysis of the 10,11-epoxide. *J Med Chem* 1987; **30**:768–773.
10 Lillibridge JH, Amore BM, Slattery JT, Kalhorn TF, Nelson SD, Finnell RH, *et al.* Protein-reactive metabolites of carbamazepine in mouse liver microsomes. *Drug Metab Dispos* 1996; **24**:509–514.
11 Gaedigk A, Spielberg SP, Grant DM. Characterization of the microsomal epoxide hydrolase gene in patients with anticonvulsant adverse drug reactions. *Pharmacogenetics* 1994; **4**:142–153.
12 Green VJ, Pirmohamed M, Kitteringham NR, Gaedigk A, Grant DM, Boxer M, *et al.* Genetic analysis of microsomal epoxide hydrolase in patients with carbamazepine hypersensitivity. *Biochem Pharmacol* 1995; **50**: 1353–1359.
13 Mauri-Hellweg D, Bettens F, Mauri D, Brander C, Hunziker T, Pichler WJ. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J Immunol* 1995; **155**:462–472.
14 Nassif A, Bensussan A, Dorothee G, Mami-Chouaib F, Bachot N, Bagot M, *et al.* Drug specific cytotoxic T-cells in the skin lesions of a patient with toxic epidermal necrolysis. *J Invest Dermatol* 2002; **118**:728–733.

15 Naisbitt DJ, Britschgi M, Wong G, Farrell J, Depta JP, Chadwick DW, *et al.* Hypersensitivity reactions to carbamazepine: characterization of the specificity, phenotype, and cytokine profile of drug-specific T cell clones. *Mol Pharmacol* 2003; **63**:732–741.

16 Nassif A, Bensussan A, Boumsell L, Deniaud A, Moslehi H, Wolkenstein P, *et al.* Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells. *J Allergy Clin Immunol* 2004; **114**:1209–1215.

17 Schnyder B, Mauri-Hellweg D, Zanni M, Bettens F, Pichler WJ. Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones. *J Clin Invest* 1997; **100**:136–141.

18 Chung WH, Hung SI, Hong HS, Hsih MS, Yang LC, Ho HC, *et al.* Medical genetics: a marker for Stevens–Johnson syndrome. *Nature* 2004; **428**:486.

19 Sullivan JR, Shear NH. The drug hypersensitivity syndrome: what is the pathogenesis? *Arch Dermatol* 2001; **137**:357–364.

20 Smigielski EM, Sirotkin K, Ward M, Sherry ST. dbSNP: a database of single nucleotide polymorphisms. *Nucleic Acids Res* 2000; **28**:352–355.

21 Walsh EC, Mather KA, Schaffner SF, Farwell L, Daly MJ, Patterson N, *et al.* An integrated haplotype map of the human major histocompatibility complex. *Am J Hum Genet* 2003; **73**:580–590.

22 Pirmohamed M, Lin K, Chadwick D, Park BK. TNFalpha promoter region gene polymorphisms in carbamazepine-hypersensitive patients. *Neurology* 2001; **56**:890–896.

23 Martin AM, Nolan D, Gaudieri S, Almeida CA, Nolan R, James I, *et al.* Predisposition to abacavir hypersensitivity conferred by HLA-B*5701 and a haplotypic Hsp70-Hom variant. *Proc Natl Acad Sci USA* 2004; **101**:4180–4185.

24 Middleton D. Current and emerging technology for HLA typing. *Int J Hematol* 2002; **76 (Suppl 2)**:150–151.

25 Tilanus MGJ, Hansen JA, Hurley CK. *Technical manual of the Thirteenth International Histocompatibility Working Group (IHWG), genomic analysis of the human MHC DNA-based typing for HLA alleles and linked polymorphisms.* Seattle, WA: IHWG; 2002.

26 Hung SI, Chung WH, Liou LB, Chu CC, Lin M, Huang HP, *et al.* HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. *Proc Natl Acad Sci USA* 2005; **102**:4134–4139.

27 Chu CC, Lin M, Nakajima F, Lee HL, Chang SL, Juji T, *et al.* Diversity of HLA among Taiwan's indigenous tribes and the Ivatans in the Philippines. *Tissue Antigens* 2001; **58**:9–18.

28 Schaid DJ, Jacobsen SJ. Biased tests of association: comparisons of allele frequencies when departing from Hardy–Weinberg proportions. *Am J Epidemiol* 1999; **149**:706–711.

29 Haldane JB. The estimation and significance of the logarithm of a ratio of frequencies. *Ann Hum Genet* 1956; **20**:309–311.

30 Pan WH, Fann CSJ, Wu JY, Hung SI, Hung YT, Chen YJ, *et al. Establishment of Taiwan Han Chinese cell and gene bank*: comparing SNP profiles in MHC region with Caucasians. Fifty-Fourth Annual Meeting of the American Society of Human Genetics; Toronto, Canada; 2004: abstract 1149.

31 Hung SI, Chung WH, Chen YT. HLA-B genotyping to detect carbamazepine-induced Stevens–Johnson syndrome: implications for personalizing medicine. *Personal Med* 2005; **2**:225–237.

32 Roujeau JC, Kelly JP, Naldi L, Rzany B, Stern RS, Anderson T, *et al.* Medication use and the risk of Stevens–Johnson syndrome or toxic epidermal necrolysis. *N Engl J Med* 1995; **333**:1600–1607.

33 Horton R, Wilming L, Rand V, Lovering RC, Bruford EA, Khodiyar VK, *et al.* Gene map of the extended human MHC. *Nat Rev Genet* 2004; **5**:889–899.

34 Pichler WJ. Modes of presentation of chemical neoantigens to the immune system. *Toxicology* 2002; **181–182**:49–54.

35 Mallal S, Nolan D, Witt C, Masel G, Martin AM, Moore C, *et al.* Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *Lancet* 2002; **359**:727–732.